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(54) Title: RECOMBINANT VACCINES AGAINST INFECTIOUS BURSAL DISEASE VIRUS

(57) Abstract: There is provided a stable vaccine for providing protection against disease having viral proteins transgenically expressed in plant cells. Also provided is a stable vaccine that provides protection against disease containing viral protein and coding sequences cloned into an *E. Coli* expression system. A method of vaccination by transgenically expressing viral proteins capable of providing protection against disease into plant cells and administering the plant cells to an animal in need of vaccination is also provided. Also provided is a method of vaccination by cloning viral protein and coding sequences capable of providing protection against disease into an *E. Coli* expression system and administering the *E. Coli* to the animal in need of vaccination.

RECOMBINANT VACCINES AGAINST INFECTIOUS BURSAL DISEASE VIRUS

FIELD OF THE INVENTION

This invention provides a transgenic plant and/or bacteria containing empty particles of Infectious Bursal Disease Virus (IBDV). This invention further provides a vaccine for protecting an avian against IBDV which comprises a transgenic plant or a bacteria containing empty particles of IBDV. This invention further provides a method for protecting an avian against IBDV comprising a transgenic plant or bacteria containing empty particles of IBDV.

BACKGROUND OF THE INVENTION

The vast majority of agents of infection in vertebrates enter the host across a mucosal surface, including generally the mucosa of the alimentary canal (including oral mucosa), the respiratory tract (including olfactory and conjunctival mucosa), the mammary glands, and the genitourinary tract. The common mucosal immune system, by way of the secretory immunoglobulin response, provides a first line of resistance to infection across mucosal surfaces in vertebrates [J. Mestecky, "The common mucosal immune system and current strategies for induction of immune responses in external secretions," *J. Clin. Immunol.*, 7, (1987) pp. 265-75].

The mucosal immune system consists of molecules, cells, and organized lymphoid structures intended to provide immunity to pathogens that invade mucosal surfaces. Mucosal infection by intracellular pathogens results in the induction of cell-mediated immunity, as manifested by CD4-positive (CD4+) T helper-type 1 cells, as well as CD8+ cytotoxic T-lymphocytes. These responses are normally accompanied by the synthesis of secretory immunoglobulin A (S-IgA) antibodies, which provide an important first line of defense against invasion of deeper tissues by these pathogens. [For a more complete review of the mucosal immune system, see Strober and James, "The mucosal immune system", *Basic & Clinical Immunology,* 8th edition, eds. Stites, Terr, Parslow, (Appleton & Lange), 1994, pp. 541-551, incorporated by reference in its entirety].

Vaccines are administered to humans and animals to induce their immune systems to produce antibodies against viruses, bacteria, and other types of pathogenic organisms. In the economically advanced countries of the world, vaccines have brought many diseases under control. Vaccines that stimulate the mucosal immune system can be used to immunize hosts, generally via an oral route, against pathogens that are transmitted via a mucosal surface. [See for example Shalaby, "Development of oral vaccines to stimulate mucosal and systemic immunity: barriers and novel strategies", *Clin. Immunol. Immunopathol.*, 74(2), 1995, pp. 127-134; Mestecky et al, "Mucosal immunity and strategies for novel microbial vaccines", *Acto Paediatr Jpn*, 36(5), 1994, pp. 537-44; and United States Patents 5,518,725 and 5,417,986.]

Infectious Bursal Disease (IBD), or Gumboro Disease, is a highly contagious viral disease of young chickens which is characterized by the destruction of lymphoid follicles in the bursa of Fabricius. Symptoms include depression, diarrhea, muscular hemorrhage, necrosis of the bursa and severe damage to the immune system. Mortality of infected birds is high, and survivors exhibit slow growth and high susceptibility to other infectious diseases. The disease attacks young chickens, usually up to 6 weeks of age. Susceptible chickens less than three weeks old do not exhibit outward clinical signs of the disease but have a marked infection characterized by gross lesions of the bursa.

Infectious Bursal Disease Virus (IBDV) is the causative agent of IBD. IBDV is a pathogen of major economic importance to the world's poultry industries. The virus belongs to the genus Avibirnavirus of the family Birnaviridae. Members of this family contain a genome composed of two double-stranded (ds) RNA segments designated A and B. The smaller genome segment B (approximately 2.8 kbp) encodes VP1, the putative virion-associated RNA polymerase, whereas the larger genome segment A (approximately 3.3 kbp) encodes VP2, VP3, and VP4 as a precursor polyprotein. The genome segment A sequence also has a small open reading frame (ORF) overlapping the 5' end of the polyprotein ORF in a different reading frame and is capable of encoding a 17 kDa polypeptide. The function of this protein remains unknown although the polypeptide is made in IBDV-infected cells and has been designated VP5. VP4 has been found to be a protease that is responsible for

processing of the precursor polyprotein into VPX (the precursor of VP2), VP3 and VP4. The major capsid protein, VP2, is the main host-protective antigen of IBDV. VP2 and VP3 are the structural proteins of IBDV and are necessary for virion assembly.

Several vaccines against IBDV are available, and others have been attempted as detailed in the prior art. The costs of these vaccines makes their use impractical, particularly in developing parts of the world. In addition, several of the vaccines require sophisticated storage and administration, which again presents problems for use in many parts of the world. Another problem with the conventional vaccines (attenuated or killed viruses) is the possibility of reconstitution of virulence of the immunizing agents.

In addition, recombinant vaccines against IBDV have been used that are based on immunization with only one recombinant IBDV protein. It has been found, in many cases, that immunization with only one recombinant viral protein, even with the most immunogenic one, does not induce the necessary protection state. Only immunization with fully assembled virion can induce immunization.

Thus there is a need for a vaccine against IBDV that is safe, inexpensive, easy to administer, and which induces the necessary protection state against IBDV.

SUMMARY OF THE INVENTION

The present invention provides a transgenic plant comprising empty particles of Infectious Bursal Disease Virus (IBDV). In one embodiment, the empty particles of the IBDV comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), and/or virion protein 4 (VP4). In one embodiment, the plant is a tobacco plant. In one embodiment, the plant is a potato plant.

The present invention further provides a vaccine for protecting an avian against IBDV that comprises a plant having an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the plant is a tobacco plant. In one embodiment, the plant is a potato plant.

The present invention further provides a bacteria comprising empty particles of the IBDV. In one embodiment, the empty particles of the IBDV comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), and/or virion protein 4 (VP4). In one embodiment, the bacteria is *E. Coli.*

The present invention further provides a vaccine for protecting an avian against IBDV that comprises a bacteria having an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV

comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the bacteria is *E. Coli.*

The present invention further provides a method of immunizing an avian comprising administering to the avian a transgenic plant containing an effective amount of a vaccine comprising an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the plant is a tobacco plant. In one embodiment, the plant is a potato plant. In one embodiment, the avian is selected from the group consisting of chicken, turkey, goose, duck, pheasant, quail, pigeon and ostrich.

The present invention further provides a method of immunizing an avian comprising administering to the avian bacteria containing an effective amount of a vaccine containing an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the bacteria is *E. Coli.* In one embodiment, the avian is selected from the group consisting of chicken, turkey, goose, duck, pheasant, quail, pigeon and ostrich.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the appended Figures in which:

FIGURE 1: is a schematic representation of the PCR strategy for amplifying the entire IBDV fragment A genome. The size of the fragment is scaled in the bottom line;

FIGURES 2 A-C:

are photographs depicting agarose gel electrophoresis of amplified cDNA fragments of IBDV fragment A genome, stained with ethidium bromide and viewed over a UV lamp.

FIGURES 3 A-C:

depict cloning of IBDV A genome into pET21a vector. Figure 3A shows a schematic representation of pET21a. Figure 3B is a photograph showing the agarose gel electrophoresis of amplified cDNA fragments stained with ethidium bromide and viewed over a UV lamp. Figure 3C is a schematic representation of the pKVI206 vector.

FIGURE 4: depicts the nucleotide sequence of the amplified IBDV cDNA fragment in pKVI206;

FIGURES 5 A-C:

are photographs showing the expression and processing of IBDV proteins in BL21 *E. Coli* cells. Figure 5A depicts a coomassie blue stained SDS polyacrylamide gel. Figure 5B depicts a western blot using anti-IBDV serum. Figure 5C depicts a western blot using anti-IBDV antibodies of purified IBDV proteins.

FIGURE 6: is a photograph showing the scanning electron microscope (SEM) microscopy of IPTG induced *E. Coli* cells (pKVI206-BL21) harboring empty IBDV particles (indicated by arrows);

FIGURE 7: is photograph showing the Western blot analysis of anti-IBDV antibodies in the serum of chickens vaccinated with purified recombinant empty particles.

FIGURE 8: is a schematic representation of the structure of the binary plasmid pBi203-IBDV;

FIGURES 9 A and B:

are photographs showing the RT-PCR of an IBDV internal sequence in transgenic potato plants indicating the expression of IBDV RNA in the tubers. The arrow indicates the amplified PCR product.

FIGURE 10: is a photograph of an autoradiograph demonstrating IBDV protein expression in plants.

Figure 11: is a photograph showing the scanning electron microscope (SEM) microscopy of leaf or tuber tissue from transgenic potato or tobacco plants harboring IBDV particles (indicated by arrows).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a transgenic plant comprising empty particles of Infectious Bursal Disease Virus (IBDV). In one embodiment, the empty particles of the IBDV comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), and/or virion protein 4 (VP4). In one embodiment, the plant is a tobacco plant. In one embodiment, the plant is a potato plant.

As defined herein, "empty particle" means a virus particle of the IBDV that is devoid of genetic elements and that permits replications.

In order to produce plant cells that express the assembled viral proteins, transgenic plants must be created containing therein the assembled viral proteins. For purposes of this invention, a transgenic plant is a plant expressing in at least some of the cells of the plant a recombinant viral immunogen.

The preferred method of administration of the vaccine is comprised of feeding the animals with the transgenic plants. Thus the transgenic plant of the invention, in preferred embodiments, is an edible plant. For the purpose of this invention, an edible plant or portion thereof of is one which is not toxic when injested by a mammal to be treated with the vaccine produced in the plant. The edible part of the plant is used as a dietary component, while the vaccine is administered in the process. The edible portions of the plant include the fruits,

leaves, stems, roots or seeds of said plant. In addition, particles were extracted from plant cells such as those of tobacco leaves or potato tubers and administered by injection. Any plant can be utilized which is able to be ingested by the animal which is fed the transgenic plant cells. In the preferred embodiment, tobacco or potato plants are used, because in these plants the expression is constitutive. By constitutive, it is meant that all of the organs of the plants express the antigens.

The process of creating the transgenic plants is set forth in the 15th Chapter of *Genetic Engineering of Plants*, incorporated herein by reference.

Some examples of applicable methods include, but are not limited to, growing whole plants from single cells. In this method, single cells (usually from a callus) are removed from a plant and plated out. The cellulose wall of the plant cells can be removed at this time, thus making the protoplast capable of taking up DNA. The cells are then given sufficient nutrients and plant hormones, auxins and cytokinins, for successful regeneration of an entire plant as is commonly known by one skilled in the art. The resulting plant therefore contains therein the desired DNA.

Another example of an applicable method, is the leaf disk technique. This simple technique involves making small leaf disks by punching holes in a leaf. These holes are then inoculated with a recombinant plasmid that contains both a selectable marker and a desired transgene carried by a bacterium, for

example Ti plasmid carried by the bacterium Agrobacterium tumefaciens. The wounded cells at the edge of the disc release substances that attract the Agrobacteria and cause them to transfer DNA in to the cells. Only those plant cells that integrate the appropriate DNA and express the selectable marker gene survive to proliferate and form a callus. The manipulation of growth factors supplied to the callus induces it to form shoots that subsequently root and grow into adult plants carrying the transgene.

Other useful techniques include the use of electric shock and guns for the transfer of DNA into the plant cells. Additionally, there is support for the use of viruses as vectors for introducing DNA into plant cells.

The present invention further provides a bacteria comprising empty particles of the IBDV. In one embodiment, the empty particles of the IBDV comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), and/or virion protein 4 (VP4). In one embodiment, the bacteria is *E. Coli.*

The present invention further provides a vaccine for protecting an avian against IBDV which comprises a plant having an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4) in a suitable carrier. In one embodiment, the plant is a tobacco plant. In one embodiment, the plant is a potato plant.

The present invention further provides a vaccine for protecting an avian against IBDV that comprises bacteria having an effective immunizing amount of empty particles in the IBDV. In one embodiment, the empty particles of IBDV comprises virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the bacteria is *E. Coli.*

For the purposes of this invention, a vaccine is a composition which, when contacted with a mammal, is capable of eliciting an immune response.

In one embodiment, the empty particles of IBDV comprise Fragment A of IBDV, which encodes for virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4), in a suitable carrier. The entire expressed assembled fragment A containing the viral proteins VP2, VP3 and VP4 is expressed in either the plant or bacterial expression vector, rather than the subunit vaccine comprising individual proteins VP2, VP3 or VP4. It has been established previously that in many cases immunization with only one recombinant viral protein, even with the most immunogenic one, does not necessarily induce the necessary protection state. However, it has been shown that immunization can be guaranteed with a fully assembled virion, achieved by expression of fragment A of IBDV.

In one embodiment, the suitable carrier is a vector. The term "vector" refers to an autonomously replicating DNA molecule. Other terms that are analogous to "vector" are "cloning vectors" and "vehicles". Vectors that comprise the DNA encoding for a clone of the expression system are also provided by the

present invention. The vectors can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Expression systems for vectors include bacteria, yeast, mammalian and insect cells. Plasmids and bacteriophage are examples of vectors. Plasmids are circular DNA duplexes that contain the requisite genetic machinery, such as a replication origin, and that confer beneficial characteristics to their hosts, such as resistance to antibiotics and mechanisms for recovery of the nucleic acids. In addition, plasmids contain a number of conveniently located restriction endonuclease sites into which the DNA to be cloned may be inserted. Some plasmids replicate under stringent control - once per cell division. Other plasmids are under relaxed control - they are normally present in 10 to as many as 700 copies per cell. Plasmids may be used to clone DNAs of up to 10 kb. Bacteriphages are cloning vectors that are used to clone DNAs of up to 16 kb. Chimeric phage DNA containing the desired DNA can be introduced into the host cells by infecting them with phages formed from the DNA by an in vitro packaging system. Phages can also be used to clone longer DNA inserts. Examples of other vectors include baculoviruses and retroviruses, DNA viruses, liposomes and other recombination vectors. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

In one embodiment, the vaccine comprises bacteria containing empty particles of IBDV Fragment A inserted in a vector suitable for expression in bacteria. In one embodiment, the bacteria is *E. Coli*. In one embodiment, the *E. Coli* expression vector is pET21a. The nucleic acid IBDV fragment A is

synthesized and cloned into a vector suitable for expression in bacteria, preferably *E. Coli*. Synthesis of the nucleic acid involves the design of specific primers (as set forth in the examples below), which are then used in a Large and Accurate RT-PCR (LA-RT-PCR) process for developing the IBDV fragments to be cloned into the vector. In order to express the viral protein in the expression vector, an additional fragment is cloned into the genome between the promoter and the terminator of the expression vector. The resulting plasmid is then capable of transforming bacterial cells as required for vaccination. The cDNA sequence encoding IBDV Fragment A inserted in the *E. Coli* expression vector pET21a is set for the in SEQ ID No. 1, as set forth in Figure 4.

In one embodiment, the vaccine comprises a plant containing empty particles of IBDV Fragment A inserted in a vector suitable for expression in plants. The nucleic acid IBDV fragment A is synthesized and cloned into a vector suitable for expression in plants, such as potato or tobacco, as set forth in the examples that follow.

The present invention further provides a method of immunizing an avian comprising administering to the avian a transgenic plant containing an effective amount of a vaccine comprising an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the plant is a tobacco plant. In one embodiment, the plant is a

potato plant. In one embodiment, the avian is selected from the group consisting of chicken, turkey, goose, duck, pheasant, quail, pigeon and ostrich.

The present invention further provides a method of immunizing an avian comprising administering to the avian bacteria containing an effective amount of a vaccine containing an effective immunizing amount of empty particles of IBDV. In one embodiment, the empty particles of IBDV comprise virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4). In one embodiment, the bacteria is *E. Coli.* In one embodiment, the avian is selected from the group consisting of chicken, turkey, goose, duck, pheasant, quail, pigeon and ostrich.

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, (Cold Springs Harbor Laboratory, New York), 1989, 1992; and in Ausubel et al., *Current Protocols in Molecular Biology*, (John Wiley and Sons, Baltimore, Maryland), 1989. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide to Methods And Applications*, (Academic Press, San Diego, CA), 1990. Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, were performed as generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual,* (Cold Springs Harbor Laboratory Press), and methods set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 were used and are incorporated

herein by reference. In-situ (In-cell) PCR in combination with Flow Cytometry was used for detection of cells containing specific DNA and mRNA sequences (Testoni et al., *Blood*, 1996, 87:3822).

General methods in immunology: Standard methods in immunology known in the art and not specifically described were generally followed as in Stites et al. (eds), *Basic and Clinical Immunology*, 8th Ed., (Appleton & Lange, Norwalk, CT), 1994, and Mishell and Shiigi (eds), *Selected Methods in Cellular Immunology*, (W.H. Freemen and Co., New York), 1980.

Immunoassays: Generally, ELISAs were the preferred immunoassays to assess specimens. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate, other immunoassays, such as radio-immunoassays (RIA) can be used as are known to those skilled in the art. Available immunoassays are extensively described in the patent and scientific literature. See for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521, as well as Sambrook et al., *Molecular Cloning: A Laboratory Manual*, (Cold Springs Harbor, New York), 1989.

Antibody production: Antibodies may be monoclonal, polyclonal or recombinant. Conveniently, the antibodies may be prepared against the

immunogen or portion thereof, for example a synthetic peptide based on the sequence, or may be prepared recombinantly by cloning techniques, or the natural gene product or portions thereof may be isolated and used as the immunogen. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), 1988, and in Borrebaeck, *Antibody Engineering - A Practical Guide*, (W.H. Freeman and Co.), 1992. Antibody fragments may also be prepared from the antibodies and include Fab, F(ab')2, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies in a host, such as a rabbit or goat, the host is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier. Antibodies coupled to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera, rendering it monospecific.

For producing monoclonal antibodies, the technique involves hyperimmunization of an appropriate donor, generally a mouse, with the immunogen and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid that has immortality and secretes the required antibody. The cells

are then cultured in bulk and the monoclonal antibodies harvested from the culture media for use.

For producing recombinant antibody [see generally Huston et al., "Protein engineering of single-chain Fv analogs and fusion proteins", pp. 46-88, and Johnson and Bird, "Construction of single-chain Fvb derivatives of monoclonal antibodies and their production in Escherichia coli", pp. 88-99, both in and *Methods in Enzymology,* 203, ed. J.J. Langone, (Academic Press, New York, NY), 1991; and Mernaugh and Mernaugh, "An overview of phage-displayed recominant antibodies", in *Molecular Methods in Plant Pathology,* eds. R.P. Singh and U.S. Singh, (CRC Press Inc., Boca Raton, FL), 1995, pp. 359-365] messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage of a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody or antibody fragment is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody can be bound to a solid support substrate, conjugated with a detectable moiety, or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice,* (Blackwell Scientific Publications, Oxford), 1982. The binding of antibodies to a solid

support substrate is also well known in the art. [For a general discussion see Harlow and Lane, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Publications, New York), 1988, and Borrebaeck, *Antibody Engineering - A Practical Guide*, (W.H. Freeman and Co.), 1992.] The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, -galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and ^{125}I.

It is noted that although examples herein refer to a nucleic acid encoding IBDV fragment A, it is apparent to one skilled in the art that the nucleic acid is replaceable by any other nucleic acid encoding an IBDV polypeptide or any other nucleic acid encoding other disease related viral proteins and still fall within the scope of the present invention.

The above discussion provides a factual basis for the use of viral proteins in creating a vaccine. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures. This section is set forth to aid in an understanding of the invention but is not intended and should not be construed to limit in any way the invention as set forth in the claims that follow thereafter.

EXAMPLES

EXAMPLE 1: Cloning and Expression of INFECTIOUS BURSAL DISEASE VIRUS (IBDV) fragment A in *E. Coli*

To be able to express the structural proteins in a way that they will be assembled in a recombinant virion, the entire coding sequence of fragment A was cloned into pET *E. Coli* expression vector.

Cloning and expression of IBDV fragment A

Two pairs of primers were designed. Primer IBDV #876 from position 15 to position 39 carries a Not I site at the 5' terminus and Nde I site (CATATG) with the first viral ATG. The reverse primer in this pair was IBDV #12 from position 1806 to 1830. The primer carries the unique Bam HI site of fragment A. The other pair is primer IBDV #18 starting from 1807 to 1831 carrying the same (fragment A) unique Bam HI site and the reverse primer IBDV# 3068 from position 3044 to 3068 containing a Not I site at the 5' prime end.

Using Large and Accurate RT-PCR (LA-RT-PCR), the fragments were developed in the lab using AMV reverse transcriptase and ex-Taq polymerase. Two fragments, 1.8 Kbp (15-1830) and 1.2 Kbp (1807-3068), were amplified (Figure 1 depicts a schematic representation of the PCR strategy for amplifying the entire IBDV A genome) and cloned into T/A cloning vector pTargeT (Promega, Madison, WI) to create pIBDV1.2 and pIBDV1.8, taking advantage of the fact that ex-Taq products carries A residues at each 3' ends of the amplified

fragments (Figure 2). Specifically, Figure 2A shows the amplification and cloning of the IBDV A genome. The amplification of IBDV genome is shown as follows: Lane 1, the 1.8 kbp of IBDV PCR product; Lane 2, the 1.2 kbp of IBDV PCR product, Lane M. 1 kb ladder. Additionally, in Figure 2B are shown the clones of the IBDV fragments after digestion by Not I-Bam HI as follows: Lane 1, pIBDV1.2 containing 1.2 kbp fragment; Lanes 2-4, Clones pIBDV1.8 carrying the 1.8 kbp fragment. Finally, in Figure 2C are depicted the clones of pKVI201 203 and 204 bearing the 3.1 kbp IBDV fragment after Not I-Bam HI digestion. More specifically, in Figure 2C Lanes 1-3 have arrows at the left side of each of the figures indicating IBDV cDNA fragments.

Following cloning, the 1.2 kbp fragment was subcloned from pIBDV1.2 by Not I-Bam HI digestion into the same sites of pIBDV1.8 to create the pKVI201 (Figure 2C). This plasmid carries the complete coding sequences of IBDV fragment A. To ensure accuracy, the entire clone was sequenced and proved to be one uninterrupted large open reading frame (Figure 3). More specifically, Figure 3 depicts the cloning of the IBDV A genome into pET21a vector. Figure 3A shows a schematic representation of pET21a. Figure 3B is a photograph of a gel wherein Lanes 1, 2 are the vectors pKVI 206, 207 after Xba I-Not I digestion. Finally, Figure 3C is a schematic representation of pKVI206 vector.

To express viral protein in *E. Coli* cells, a 3.1 kbp Nde I-Not I fragment starting from the first viral ATG was cloned into Nde I-Not I sites in between the T7 promoter and the terminator of pET21a. The newly constructed plasmid

pKVI206 was used to transform BL21 cells carrying the T7 polymerase gene under the IPTG induceable lac Z promoter (Figure 4, a nucleotide sequence of the IBDV cDNA fragment in pKVI206).

To show viral protein expression and processing, BL21 cells carrying the pKBVI206 plasmids were treated with 1mM IPTG for 14 hours at room temperature and the cell extracts were subjected to SDS polyacrylamide gel electrophoresis. From the coomassie blue staining one could detect a bend of 32 kDa similar in size to the mature IBDV VP3 (Figure 5A, lane 2). This band does not appear in the control BL21 cells carrying pET21a and in uninduced BL21-pKVI206 (Figure 5A, Lanes 1 and 3 respectively).

To show conclusively that the newly expressed protein is IBDV protein, Western analysis using anti-IBDV serum from an infected chicken were performed. Induction of 45, 32 and 28 kDa proteins from BL21-pKVI206 cells reacted positively with anti-IBDV antiserum (Figure 5B Lane 3). Similar 32 kDa VP3 from infected bursa that was used as positive control could be detected (Figure 5B Lane 2 containing IPTG induced pKVI206-BL21 cells). Samples from extracts of the negative control did not react with the antiserum (Figure 5B Lanes 1 and 4; uninfected bursa tissue and IPTG induced pET21a-BL21 cells respectively). The presence of the mature viral proteins in the induced BL21-pKVI206 cells is an indication that the large IBDV polyprotein was processed into mature proteins as was expected.

Immuno-electron-microscopy analysis revealed virion-like structures in the induced cells following expression. Electron microscopy grids covered with anti-IBDV serum were used to capture viral proteins and the same serum was used to decorate the captured virions. In extracts taken from induced BL21 pKVI206, virion structures were present which appeared to be similar to those virion structures found in IBDV infected bursal cells (Figure 6).

For testing the immunogenicity, recombinant empty virions were purified using Affi gel Hz (BioRad) affinity chromotography. Chicken anti-IBDV antibodies were coupled to the matrix and induced *E. Coli* and IBDV infected bursa extracts were used to purify the viral proteins. It was demonstrated that IBDV proteins were purified, as can be seen by the presence of the 32 kDa VP3 in a coomassie blue stained polyacrylamide gel (PAGE), Western analysis (Figure 5C) and viral particles in immuno-electron microscopy (Figure 6).

Results of Administration of Vaccine

To test recombinant viral empty particles immunogenicity, a vaccine comprising purified viral particles oil adjuvant was used to vaccinate young chicks.

Groups of 10-12 chickens were injected with viral empty particles purified from BL21-pKVI206 cells, viral particles purified from infected bursa, New Gamburite (Shafit's commercial vaccine), and column purified extract from BL21-pET21a cells. The chickens were vaccinated again three weeks later as a

booster and three weeks after that the chickens were challenged with a virulent IBDV virus isolated from the bursa of young infected chickens. Before-challenge samples were analyzed by monitoring the induction of anti-IBDV antibodies.

In 75% of the tested chickens vaccinated with recombinant empty particles, antibodies could be detected as tested by Western analysis (Figure 7). Testing included a Western blot analysis of anti-IBDV antibodies in the serum of vaccinated chicken with purified recombinant empty IBDV particles. The proteins from the pKVI206-BL21 cells were electrophoresed on SDS-PAGE and subsequently blotted onto a nitrocellulose sheet. The lanes on the blot were separated, and each was reacted with the serum of a different chicken as the source of primary antibodies (Lanes 1-4).

When challenged, 10 out of 11 chickens of the group that was immunized with the recombinant vaccine survived and did not show any disease symptoms (Table 1). Moreover, viral antigen was not detected in any of the chickens in the group, indicating the inability of the challenged virus to replicate in the chickens vaccinated with the recombinant vaccine. The cause of death of the one chicken in the immunized group that died was not related to IBDV infection, since viral antigens could not be detect in the serum of the dead chicken (Table 1).

Table 1:

Vaccine	Bursa AGP	Av. Weight (gr)	Death following exposure
Gumborit® 422019 (18-2-99)	-	3.49	0/10
Non vaccinated control	+	1.24	5/12
2 X LB (14-1-99) & (2-2-99)	-	1.24	1/11

Nine out of 11 chickens vaccinated with extracts from BL21-pET21a cells and 4 out of 10 of the control, non-vaccinated chickens, died after being challenged. Viral antigens were found in all chickens of the two groups, indicating virus replication. In the positive control group, in which the chickens were vaccinated with "Shafit" commercial vaccine, none of the chickens showed any disease symptoms and no virus antigen was found (Table 1).

EXAMPLE 2

Expression of IBDV particles in potato (Solanum tuberosum) and tobacco (Nicotiana tabacum) plants

Plasmid construction

The plasmid pBIN m-gfp5-ER, carrying a modified GFP gene, was obtained from Dr Jim Haseloff, [Haseloff et al., "Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly", *Proc. Nat'l. Acad. Sci. USA* 94, 1997, pp. 2122-2127]. The polylinker of pBluescipt KS and the GUS gene were introduced in place of GFP, and the plasmid was named p103.

The GUS gene was pulled out of p103 by digesting with XbaI and SacI. The sticky ends of the vector were made blunt with T4 - DNA polymerase and re-ligated. This plasmid was named p203. An EcoRI fragment (IBDV genes VP2, VP3 3.2 Kb) was cloned from the bursa of infected chickens by RT- PCR . The gene was inserted into the binary vector p203 at the EcoRI site, downstream from the 35S constitutive (CaMV) promoter (Figure 8).

The engineered binary plasmid was named pBi203-IBDV. The orientation of the IBDV genes in the engineered binary plasmid was examined by PCR, colony hybridization and sequencing.

The plasmid pBi203-IBDV was introduced into the Agrobacterium tumefaciens strain LBA4404 by electroporation (Bio-Rad Gene Pulser II apparatus, cuvette gap 0.2 cm, voltage 2.5kV Capacitor 25 F. Resistor 200 Time Constant 4-5 msec). The electroporated bacteria were subsequently selected by PCR and by selection with the antibiotic rifampicin.

Plant transformation

Leaf disks of tobacco plants (Nicotiana tabacum cv. SR1 or NN) or mini tuber disks from potatoes (Solanum tuberosum cv. Desiree) were transformed according to the technique detailed in Horsch et al. ["RT: A simple and general method for transferring genes into plants", *Science* 227, 1985, pp. 1229-1231] for tobacco plants and as described by Snyder and Belknap, ["A modified method for routine Agrobacterium mediated transformatino of in vitro grown potato microtubers", *Plant Cell Reports* 12, 1993, pp. 324-327] with the transfected A. tumefaciens harboring p203Bi-IBDV and incubated on a regeneration medium composed of MS salt and vitamin mixture (M0222 Duchefa) supplemented with 2% sucrose, 1% mannitol, 2mg/l zeatin, 0.1 mg/l indoleacetic acid, 300 mg/l kanamycin (Km) and 1% Noble agar (pH 5.8). Regenerating shoots were transferred individually to a medium containing MS salt and vitamin mixture, 3% sucrose, 300 mg/l of Km and 1% Noble agar (pH 5.8).

Transgenic rooted plantlets were transferred to soil and grown in a greenhouse at 25°C under a 16-hours light / 8-hours dark daily cycle. These plants were also self-pollinated, and their seeds were collected and germinated on Km-containing MS medium to have R1 generation.

DNA extraction and PCR reaction

Twenty two independent adult transgenic plants were used for PCR. DNA was extracted from fresh tissue, as described by Edwards, et al, ["A simple and rapid method for the preparation of plant genomic DNA for PCR analysis", *Nucleic-Acids-Research* 19:6, 1991, p. 1349; 3 ref. Plant Biotechnology Section, ICI Seeds, (Jealott's Hill Research Station , Bracknell, Berkshire, UK)]. The PCR reaction mixture (25 μl) consisted of 25 mM Tris HCl, pH 9.3, 50 mM KCl, 2 μl $MgCl_2$, 1mM β-mercapthoethanol, 50 μM of each of the four dNTPs, 150 ng of each primer: primers for VP2: sense #93,and antisense # 92 (Stram et al., "Applications of the polymerase chain reaction to detect infection Bursal Disease Virus in natuarlly infected chickens", *Avian Diseases* 38, 1994, pp. 879-884), DNA template 150-300 ng, 2.5 units of Taq DNA polymerase (Advanced Biotechnology). The reaction mixture was subjected to initial denaturation of the genomic DNA at 94° C for 10 minutes, followed by 1 minute at 56° C. The reaction mixture was then incubated for 2 minutes at 72° C and further subjected to 40 PCR cycles (each consisting of 30 seconds at 92° C, 30 seconds at 55° C, and 30 seconds at 72°C), concluding with a 7 minutes incubation at 72°C.

RT-PCR

Total RNA was extracted from transgenic plants leaves (for tobacco) .and tubers (for potatoes) (Tri reagent, Sigma). RNA (2μg) was taken for reverse transcription (RT) reaction in a final volume of 20 μl using AMV reverse transcriptase (Promega) according to the manufacturer's instructions. The reaction was carried out at 42°C for 1 hour. All the RT reaction mixture served as a template for a PCR, carried out at 94°C for 10 minutes followed by 30 cycles of 94°C (30 seconds); 58°C (30 seconds); 72°C (30 seconds). The sequences of the IBDV PCR primers are as described by Stram et al. (1994), or of a NOS terminator (this primer is located on the part of Nos- terminator beyond the poly A site. Therefore it will be found on the DNA but not on the transcribed RNA). The reaction products were separated on a 1.5% agarose gel.

The reaction products are shown in Figures 9A and B, with the arrow indicating the amplified PCR product. Specifically, Figure 9A depicts in Lane 1 the positive control which is the PCR using the binary plasmids pBi203 as a template. Lanes 2-6 and 14-15 show the PCR results using transgenic potato tuber DNA from the respective lines as a template. Lane NN, the negative control, depicts the PCR product using non-transgenic potato tuber DNA as a template. Lane PBR Alu shows the DNA of molecular size markers. Regarding Figure 9B, Lane 1 is the negative control which shows the PCR product using the binary plasmids pBi203 as a template. Lanes 2-6 and 14-15 show the RT-PCR results using transgenic potato tuber RNA from the respective lines as

a template. Lane NN, the negative control, depicts the RT-PCR product using non-transgenic potato tuber RNA from lane 3 as a template, however the RT enzyme was omitted from the reaction mixture.

Western blotting

Protein was extracted from transgenic plants (tobacco or potato) in the following extraction buffer (2 ml/gr fresh tissue) [Edelbaum et al,, "Two antiviral proteins from tobacco: Purification and characterization by monoclonal antibodies to human SYMBOL 98\f "Symbol\s 12 – interferon", *Proc. National Academy Sci. USA* 87, 1990, pp. 588-592]. 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 7 mM Mg acetate, 150 mM glucose, 30 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, 100 μM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol and 0.5%(v/v) NP-40. The homogenate was centrifuged at 15,000 g, 4° C for 15 minutes and the supernatant fluid was aliquoted and kept at -80° C. Protein content was determined according to Bradford ["A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding", *Anal. Biochem.* 72, 1976, pp. 248-254]. Proteins were electrophoresed in 12.5% SDS-polyacrylamide gels according to Laemmli ["Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature* 227, 1970, pp. 680-685] and electroblotted onto nitrocellulose membranes according to Towbin et al. ["Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications:", *Proc. Nat'l. Acad. Sci. USA*, 76, 1979, pp.4350-4354]. The membrane was incubated with Rabbit anti IBDV antiserum at a 1:100 or 1:500

dilution for 16 hours at 4° C. This was followed by a 60-minute incubation with 10^6 dpm of 125I-labeled Protein A (IM144, Amersham). Following washing, the membrane was subjected to phospho-imaging (Fuji imaging plate) followed by autoradiography. The autoradiograph was scanned by the Power Look 200 apparatus (Umax) and subjected to an integrated band-intensity-area data analysis (NIH.Image 1.61) (Figure 10). The lanes showed the following results: Lane 1 showed the molecular weight marker; Lane C1-3 show the controls of proteins from non-transgenic potatoes; Lanes 7-1-7-3 and B1 show the proteins from infected bursa; and Lane B2 shows the proteins from infected bursa diluted 1:10.

Immunosorbent electron microscopy

Leaf or tuber tissue (3-8 g) were ground in by mortar and pestle in liquid N2. Twenty μL extraction buffer containing 500 mM PO_4 buffer pH 7.4; 2 M urea, 4% PVP (w/v); 0.5% β.-mercaptoethanol were added. The extract was passed through a funnel of Miracloth filter on a disposable plastic tray to which 1 ml of 33% Triton X 100 was added. Samples were centrifuged at 12K rpm for 15 min, and the supernatant was transferred onto a tube containing 5 ml of 30% sucrose in PBS buffer pH 7.0. The tubes were centrifuged at 35,000 rpm in a Backman 52I rotor or 120 minutes at 12°C. The supernatant was discarded and the pellet was re-suspended in 50 μL of PBS buffer pH 7.4 and centrifuged at 12,000 rpm for 10 minutes. The pellet was discarded and the supernatant extracted with an equal volume of chloroform. An aliquot of 10 μl of the aqueous phase was placed on a grid coated with anti-IBDV serum and stained overnight with 2%

PTA pH 7.0. The grids were viewed in an electron microscope at a magnification of X 88,000. Figure 11 shows the scanning electron microscope (SEM) microscopy of IBDV particles in transgenic potato and tobacco plants.

The Transgenic Plants

All the transgenic tobacco and potato plants are of Ro generation. They all have flowered and set seeds.

Results of a challenge experiment

Young chicks were immunized as depicted in Table 2. When challenged, 85% of the chickens in the group that was immunized by injection with extracts from transgenic tobacco leaves, and 57% of the chickens in the group that was immunized via an oral route with recombinant potato tubers, survived. In comparison, 92% of the chicken vaccinated by injection with a commercial vaccine survived, vs. only 17% of the unvaccinated chickens. The results are summarized in Table 2.

Table 2:

Antigen	Mode of delivery	%deaths
Recombinant potato tubers	Oral delivery	43
Tobacco leaves	injection	15
Control not vaccinated		83
Control vaccinated with a commercial vaccine (Gumborit®)	injection	8

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been described hereinabove and that numerous modifications, all of which fall within the scope of the present invention, exist. Rather the scope of the invention is defined by the claims that follow:

CLAIMS

What is claimed is:

1. A transgenic plant comprising empty particles of the Infectious Bursal Disease Virus.

2. The transgenic plant of claim 1, wherein the empty particles of the Infectious Bursal Disease Virus comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), and/or virion protein 4 (VP4).

3. The transgenic plant of claim 2, wherein the plant is a tobacco or potato plant.

4. The transgenic plant of claim 3, wherein the plant is a tobacco plant.

5. The transgenic plant of claim 3, wherein the plant is potato plant.

6. A vaccine for protecting an avian against Infectious Bursal Disease Virus that comprises a plant having an effective immunizing amount of empty particles in the Infectious Bursal Disease Virus.

7. The vaccine of claim 6, wherein the empty particles of Infectious Bursal Disease Virus comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4).

8. The vaccine of claim 7, wherein the plant is a tobacco or potato plant.

9. The vaccine of claim 8, wherein the plant is a tobacco plant.

10. The vaccine of claim 8, wherein the plant is a potato plant.

11. A bacteria comprising empty particles of the Infectious Bursal Disease Virus.

12. The bacteria of claim 11, wherein the empty particles of the Infectious Bursal Disease Virus comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), and/or virion protein 4 (VP4).

13. The bacteria of claim 12, wherein the bacteria is *E. Coli.*

14. A vaccine for protecting an avian against Infectious Bursal Disease Virus that comprises a bacteria having an effective immunizing amount of empty particles in the Infectious Bursal Disease Virus.

15. The vaccine of claim 14, wherein the empty particles of Infectious Bursal Disease Virus comprise one or more of virion protein 2 (VP2), virion protein 3 (VP3), virion protein 4 (VP4).

16. The vaccine of claim 15, wherein the bacteria is *E. Coli.*

17. A method of immunizing an avian comprising administering to the avian a transgenic plant containing an effective amount of the vaccine according to claim 7.

18. A method of immunizing an avian comprising administering to the avian bacteria containing an effective amount of the vaccine according to claim 15.

19. The vaccine of any of claims 6-10 or 14-16, wherein the avian is a chicken, turkey, goose, duck, pheasant, quail, pigeon and ostrich.

20. The method of claim 17 or 18, wherein the avian is a chicken, turkey, goose, duck, pheasant, quail, pigeon and ostrich.

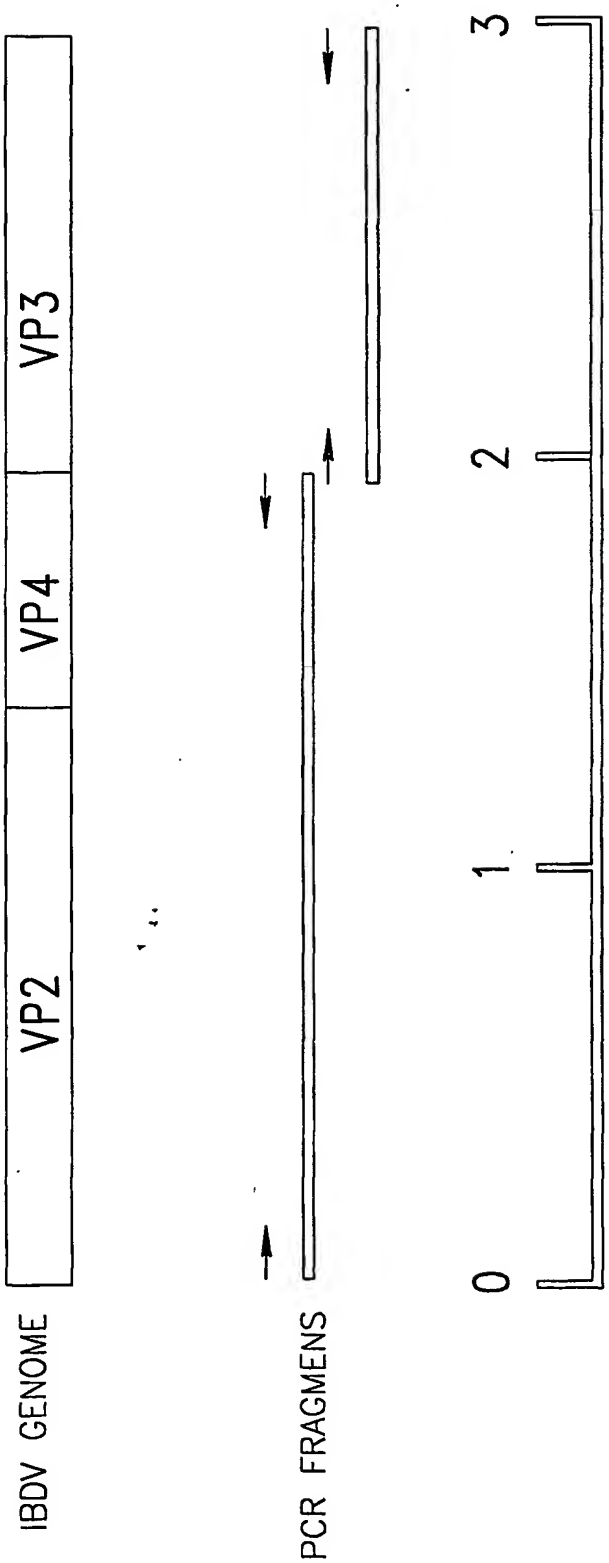

FIG.1

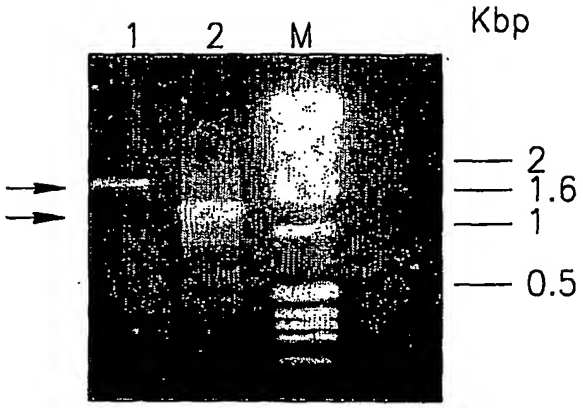

FIG.2A

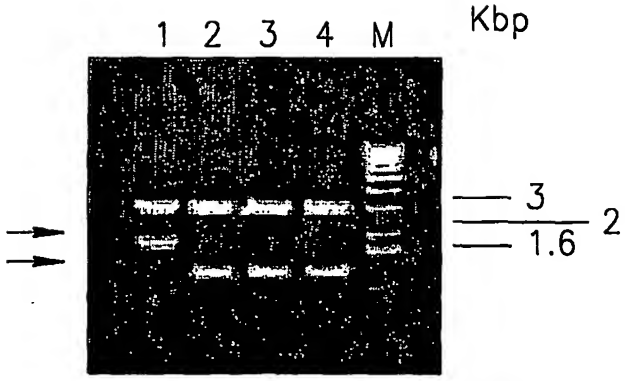

FIG.2B

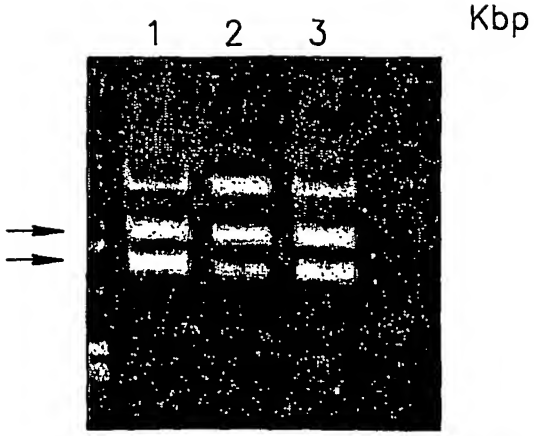

FIG.2C

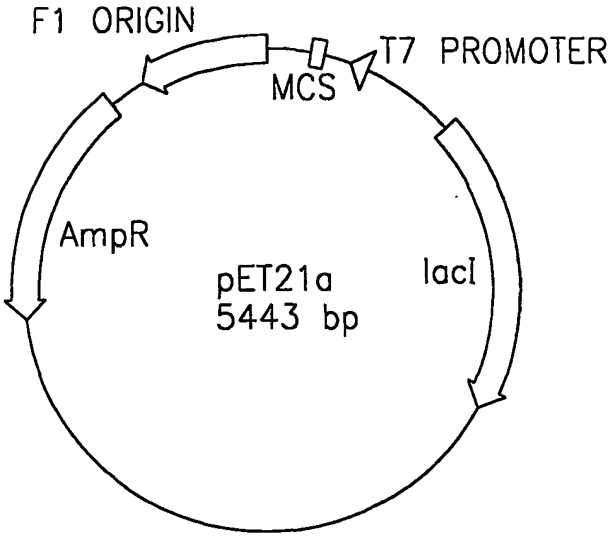

FIG.3A

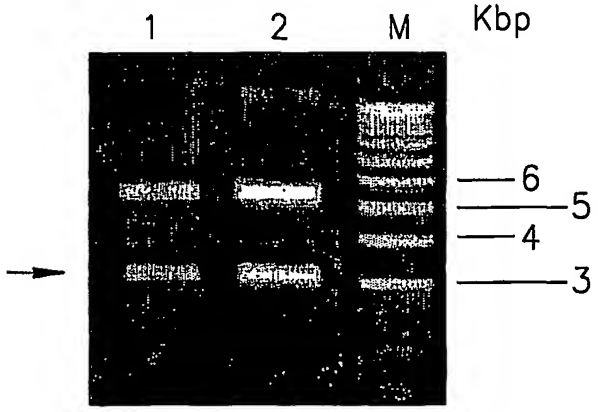

FIG.3B

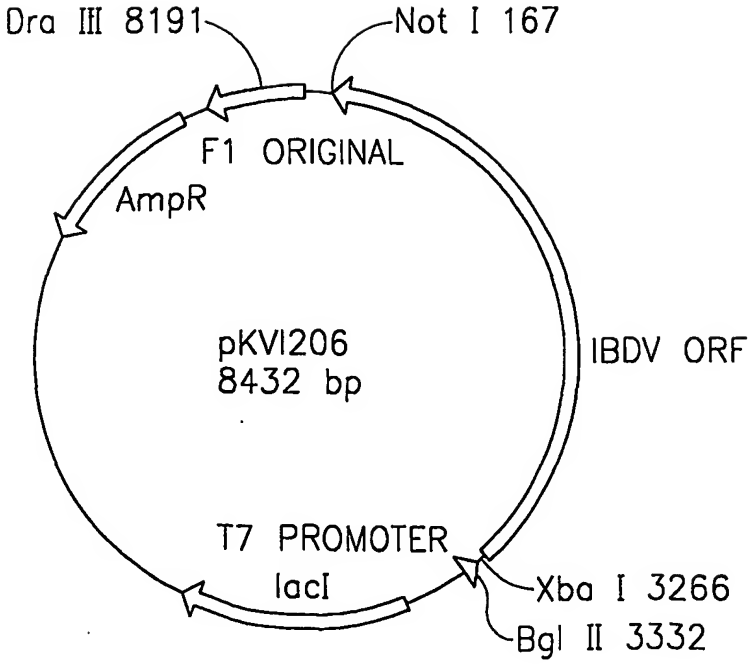

FIG.3C

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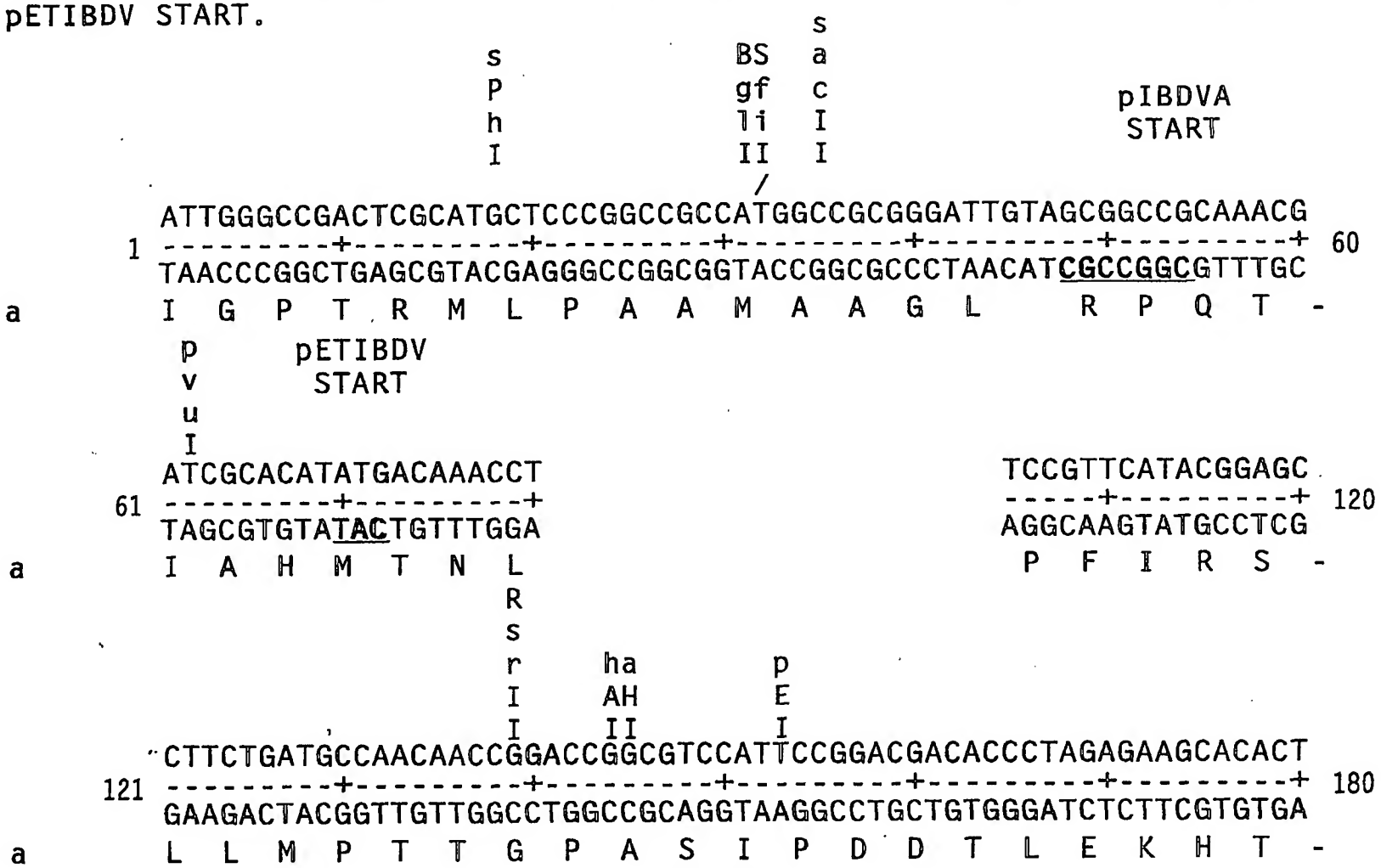

FIG.4A

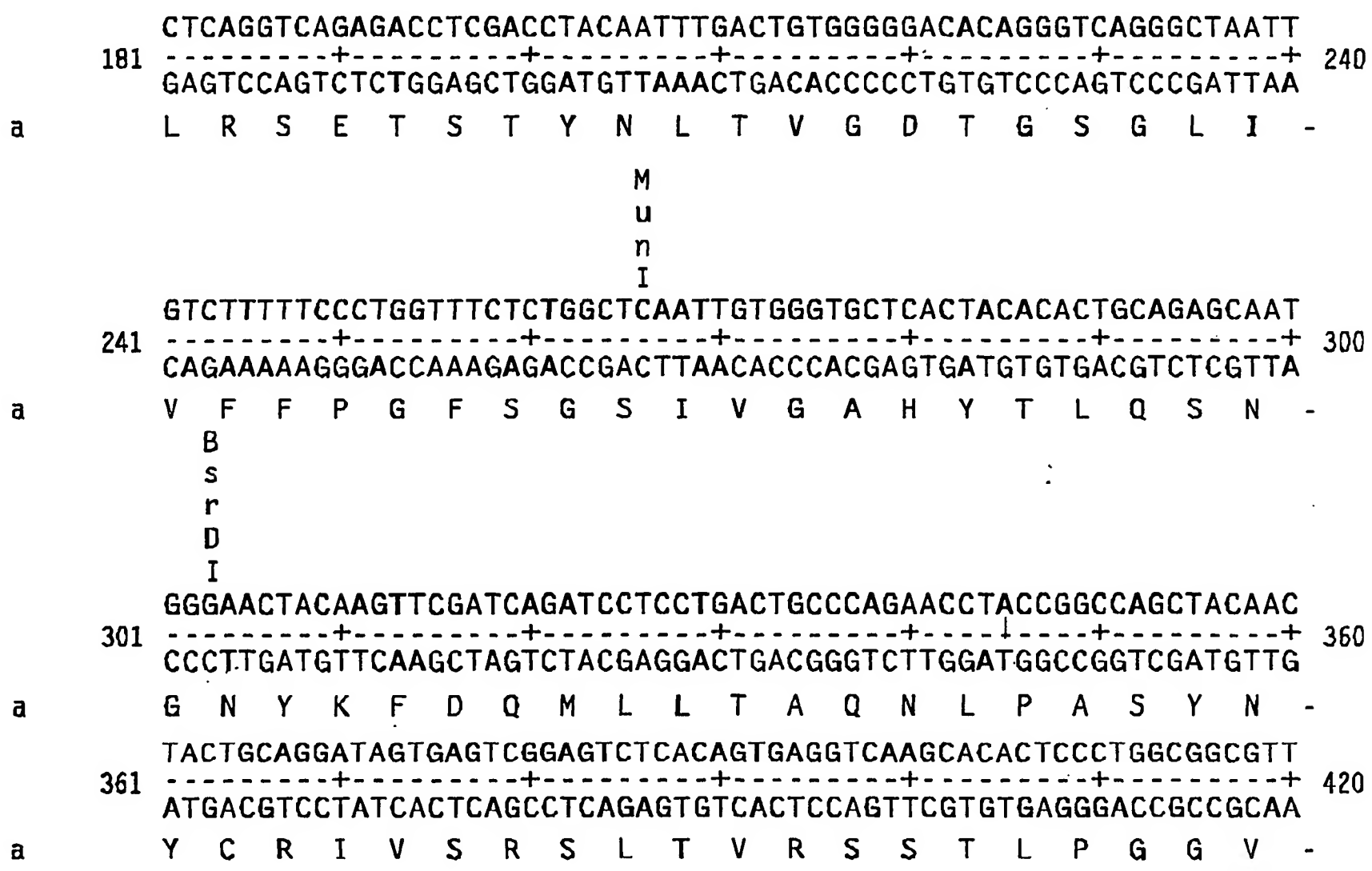
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FIG.4B

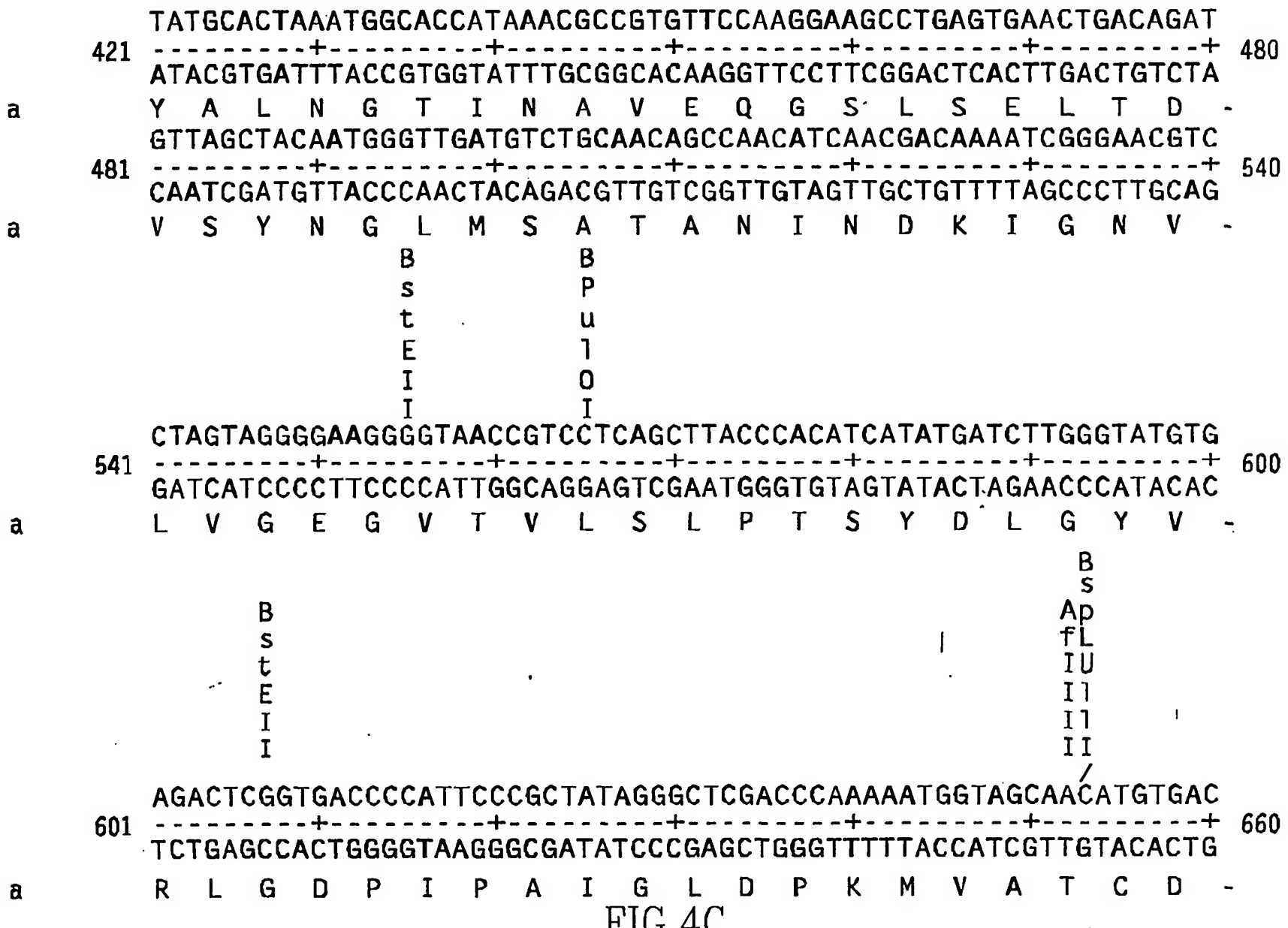
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FIG.4C

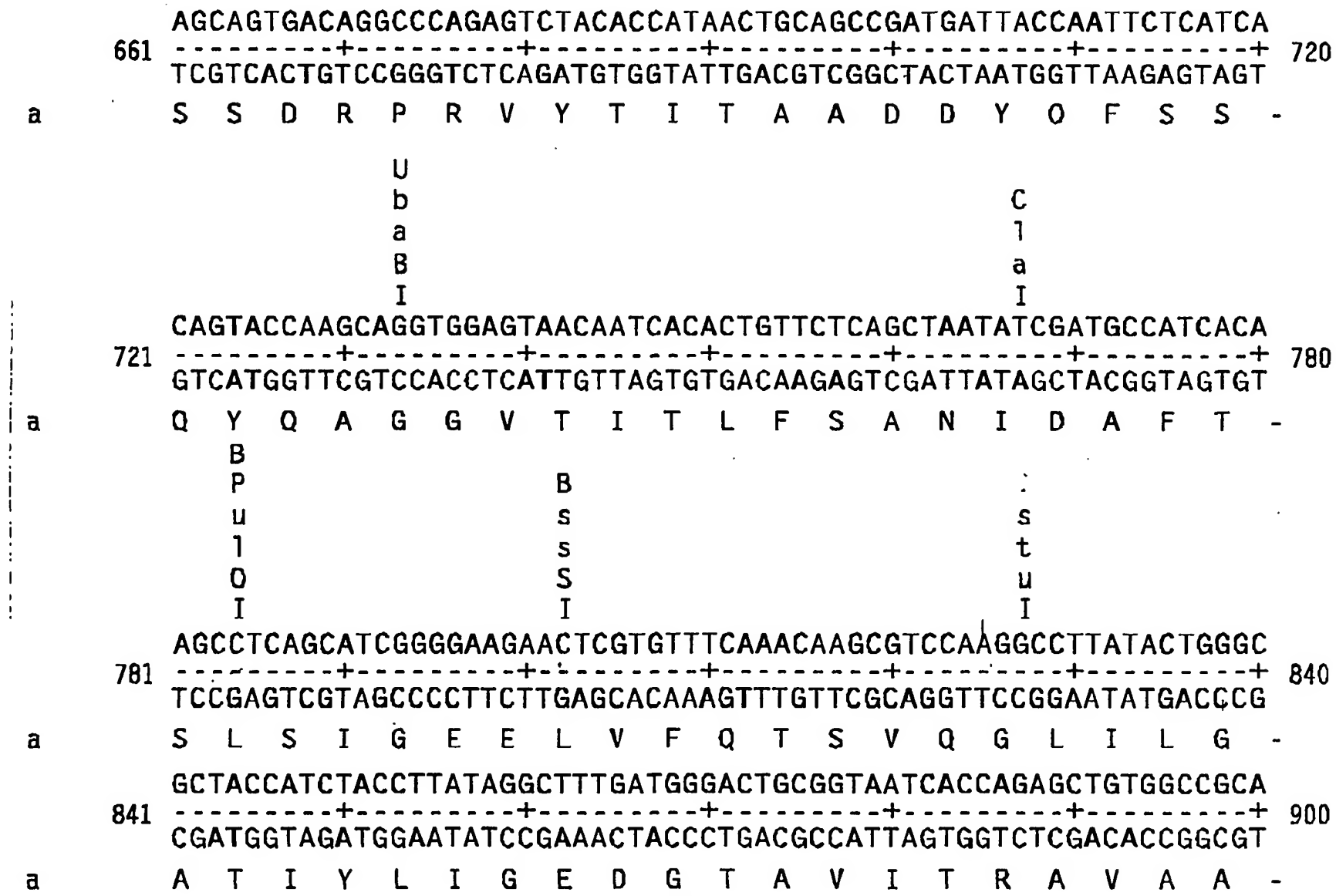
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FIG.4D

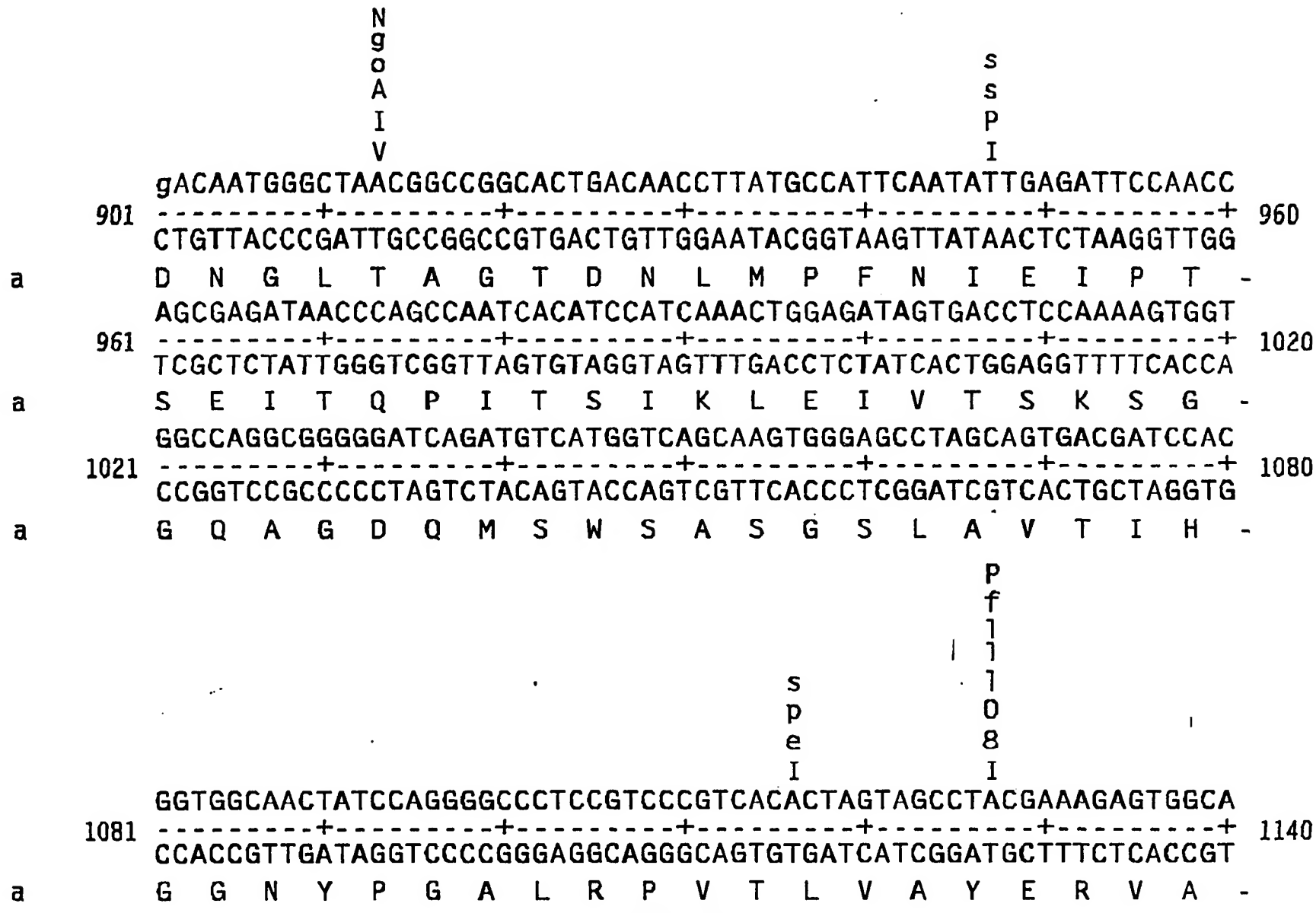
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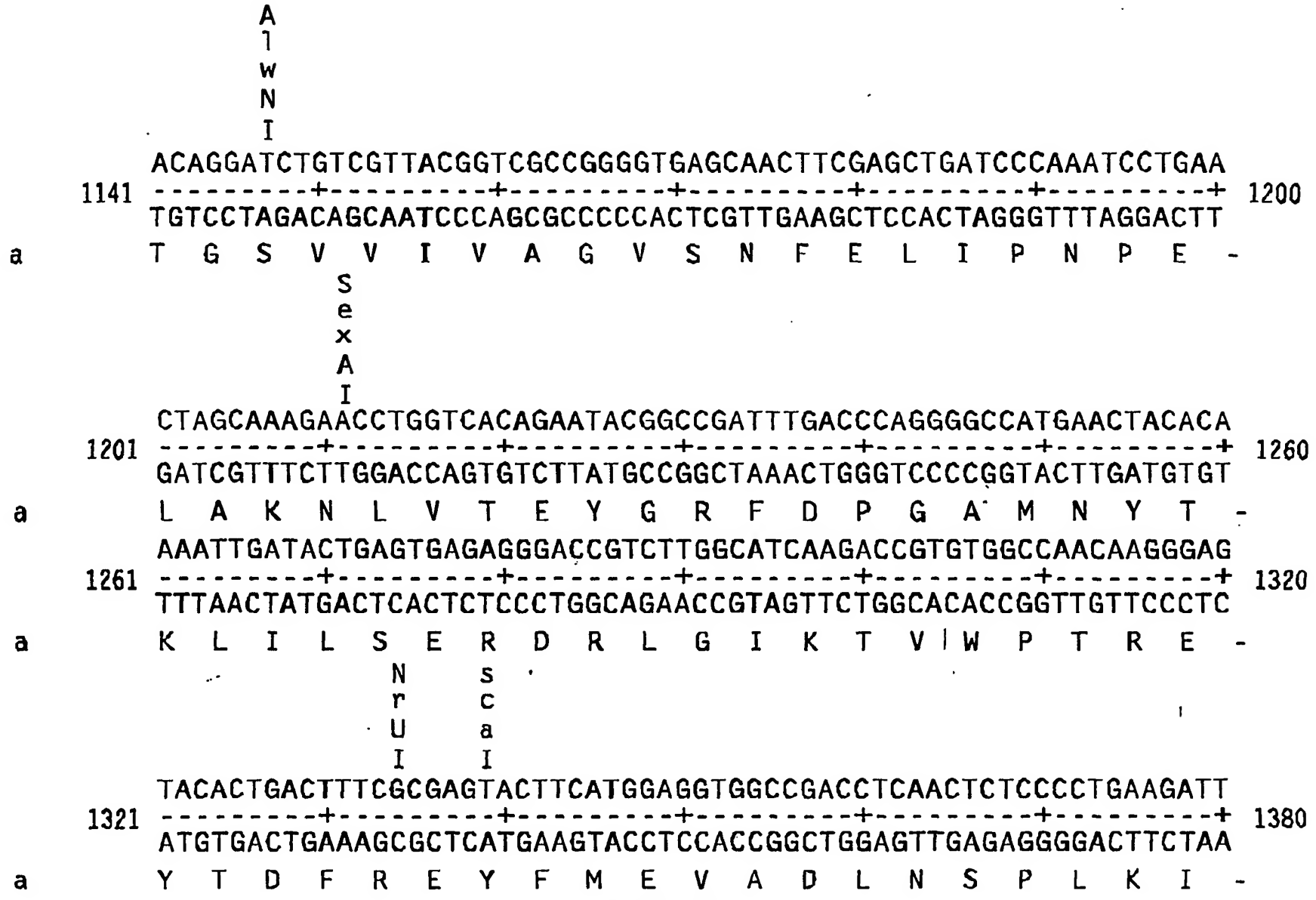
FIG.4E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a K L I L S E R D R L G I K T V W P T R E -
NruI ScaI
TACACTGACTTTCGCGAGTACTTCATGGAGGTGGCCGACCTCAACTCTCCCCTGAAGATT
1321 ---------+---------+---------+---------+---------+---------+---------+ 1380
ATGTGACTGAAAGCGCTCATGAAGTACCTCCACCGGCTGGAGTTGAGAGGGGACTTCTAA
a Y T D F R E Y F M E V A D L N S P L K I -

FIG.4F

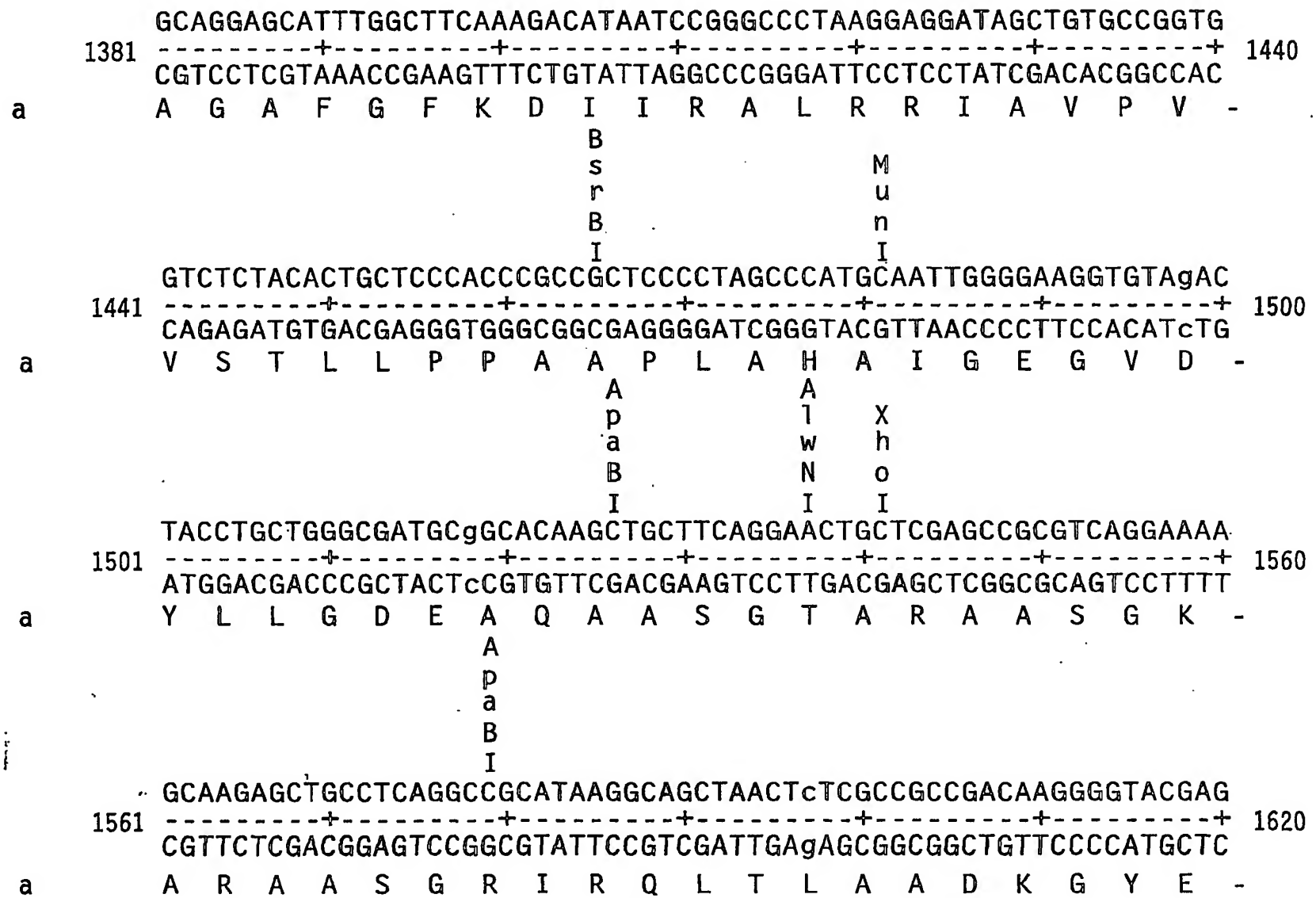

GCAGGAGCATTTGGCTTCAAAGACATAATCCGGGCCCTAAGGAGGATAGCTGTGCCGGTG
1381 ---------+---------+---------+---------+---------+---------+---------+ 1440
CGTCCTCGTAAACCGAAGTTTCTGTATTAGGCCCGGGATTCCTCCTATCGACACGGCCAC
a A G A F G F K D I I R A L R R I A V P V -
B
s M
r u
B n
I I
GTCTCTACACTGCTCCCACCCGCCGCTCCCCTAGCCCATGCAATTGGGGAAGGTGTAgAC
1441 ---------+---------+---------+---------+---------+---------+---------+ 1500
CAGAGATGTGACGAGGGTGGGCGGCGAGGGGATCGGGTACGTTAACCCCTTCCACATcTG
a V S T L L P P A A P L A H A I G E G V D -
A A
p l X
a w h
B N o
I I I
TACCTGCTGGGCGATGCgGCACAAGCTGCTTCAGGAACTGCTCGAGCCGCGTCAGGAAAA
1501 ---------+---------+---------+---------+---------+---------+---------+ 1560
ATGGACGACCCGCTACTcCGTGTTCGACGAAGTCCTTGACGAGCTCGGCGCAGTCCTTTT
a Y L L G D E A Q A A S G T A R A A S G K -
A
p
a
B
I
GCAAGAGCTGCCTCAGGCCGCATAAGGCAGCTAACTcTCGCCGCCGACAAGGGGTACGAG
1561 ---------+---------+---------+---------+---------+---------+---------+ 1620
CGTTCTCGACGGAGTCCGGCGTATTCCGTCGATTGAgAGCGGCGGCTGTTCCCCATGCTC
a A R A A S G R I R Q L T L A A D K G Y E -

FIG.4G

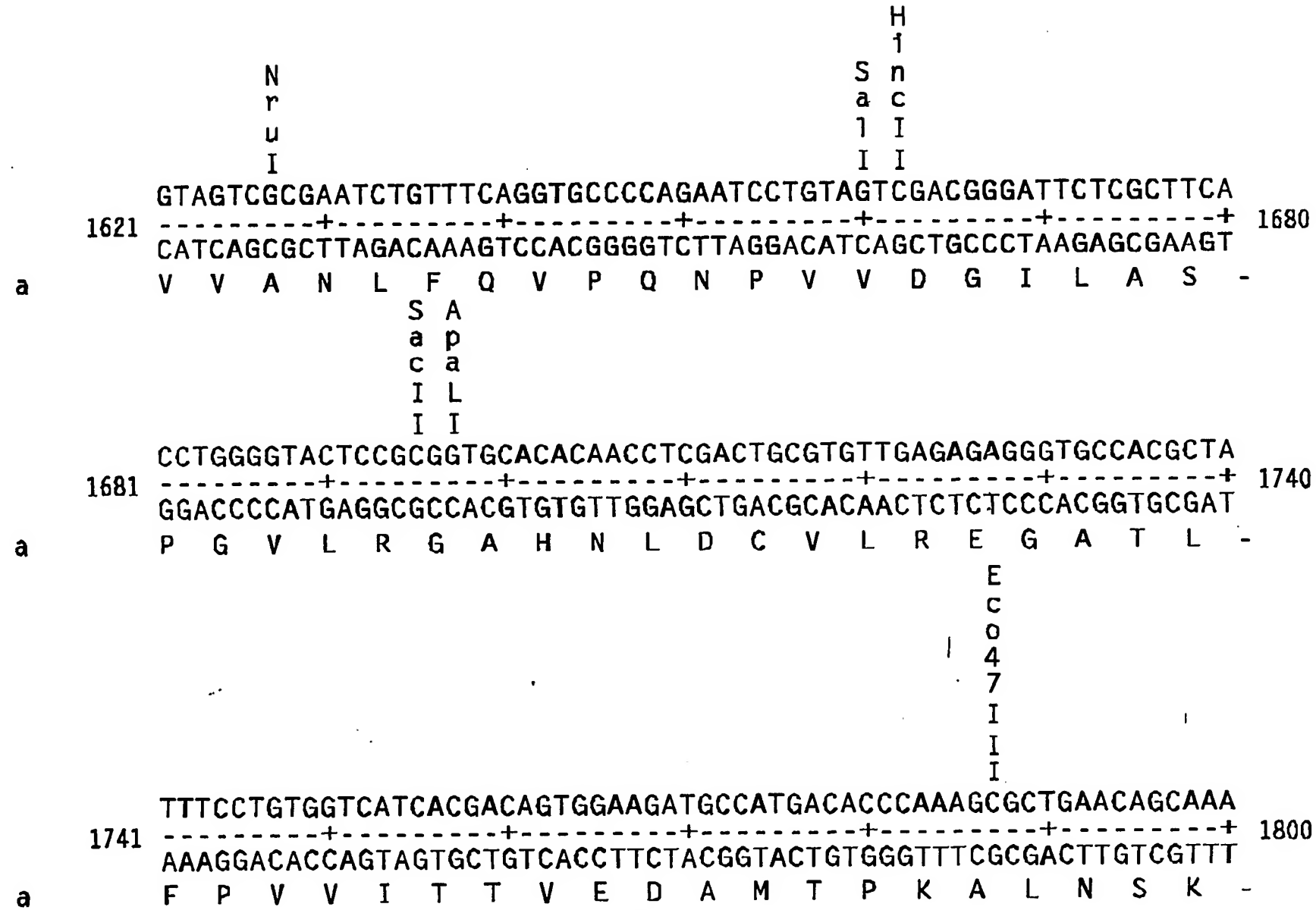

NruI
SalI
HincII
GTAGTCGCGAATCTGTTTCAGGTGCCCCAGAATCCTGTAGTCGACGGGATTCTCGCTTCA
1621 ---------+---------+---------+---------+---------+---------+---------+ 1680
CATCAGCGCTTAGACAAAGTCCACGGGGTCTTAGGACATCAGCTGCCCTAAGAGCGAAGT
a V V A N L F Q V P Q N P V V D G I L A S -
SacI
ApaLI
CCTGGGGTACTCCGCGGTGCACACAACCTCGACTGCGTGTTGAGAGAGGGTGCCACGCTA
1681 ---------+---------+---------+---------+---------+---------+---------+ 1740
GGACCCCATGAGGCGCCACGTGTGTTGGAGCTGACGCACAACTCTCTCCCACGGTGCGAT
a P G V L R G A H N L D C V L R E G A T L -
Eco47III
TTTCCTGTGGTCATCACGACAGTGGAAGATGCCATGACACCCAAAGCGCTGAACAGCAAA
1741 ---------+---------+---------+---------+---------+---------+---------+ 1800
AAAGGACACCAGTAGTGCTGTCACCTTCTACGGTACTGTGGGTTTCGCGACTTGTCGTTT
a F P V V I T T V E D A M T P K A L N S K -

FIG.4H

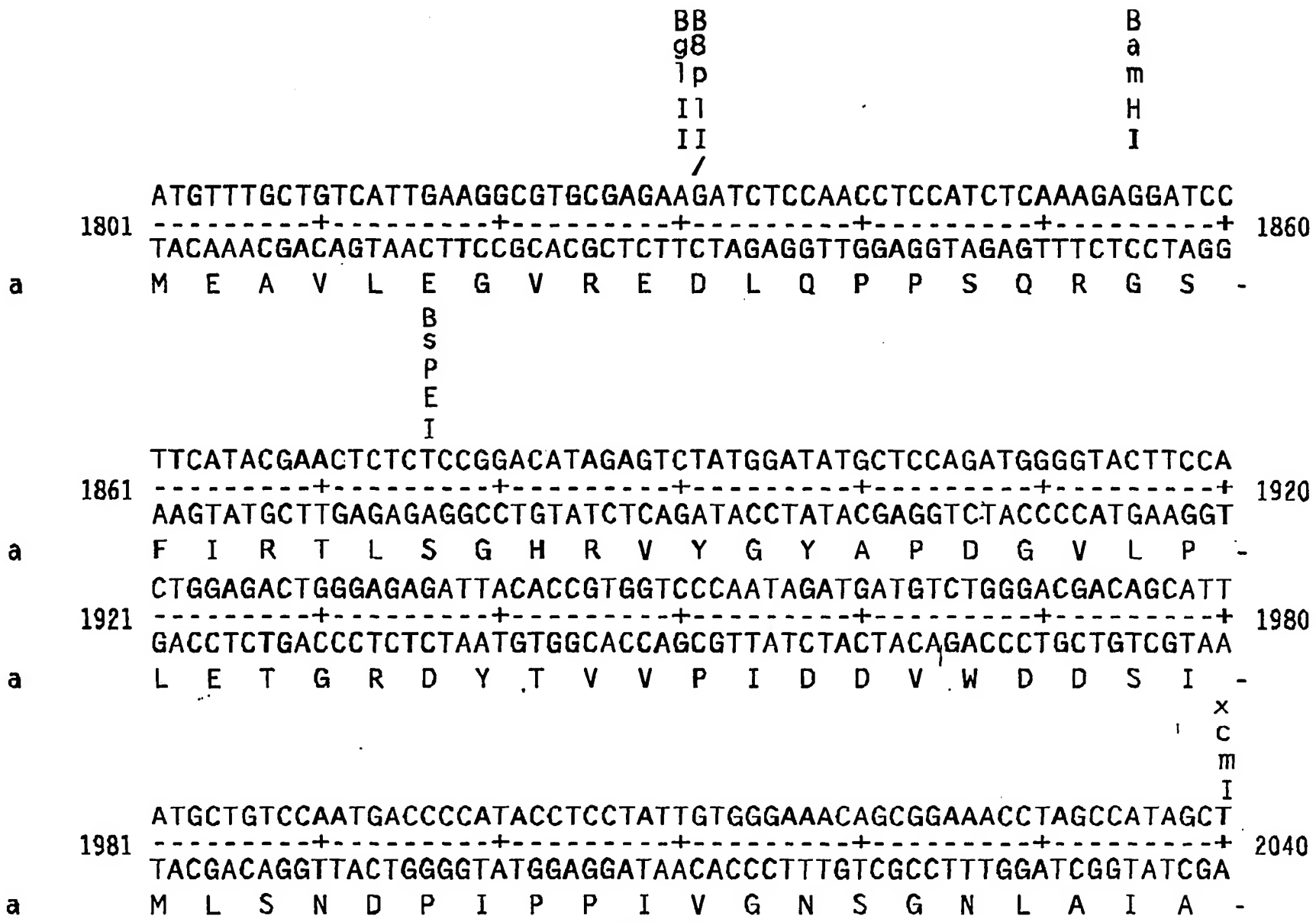

BBg8lpII II /
BamHI
ATGTTTGCTGTCATTGAAGGCGTGCGAGAAGATCTCCAACCTCCATCTCAAAGAGGATCC
1801 ---------+---------+---------+---------+---------+---------+---------+ 1860
TACAAACGACAGTAACTTCCGCACGCTCTTCTAGAGGTTGGAGGTAGAGTTTCTCCTAGG
a M E A V L E G V R E D L Q P P S Q R G S -
BspEI
TTCATACGAACTCTCTCCGGACATAGAGTCTATGGATATGCTCCAGATGGGGTACTTCCA
1861 ---------+---------+---------+---------+---------+---------+---------+ 1920
AAGTATGCTTGAGAGAGGCCTGTATCTCAGATACCTATACGAGGTCTACCCCATGAAGGT
a F I R T L S G H R V Y G Y A P D G V L P -
CTGGAGACTGGGAGAGATTACACCGTGGTCCCAATAGATGATGTCTGGGACGACAGCATT
1921 ---------+---------+---------+---------+---------+---------+---------+ 1980
GACCTCTGACCCTCTCTAATGTGGCACCAGCGTTATCTACTACAGACCCTGCTGTCGTAA
a L E T G R D Y T V V P I D D V W D D S I -
xcmI
ATGCTGTCCAATGACCCCATACCTCCTATTGTGGGAAACAGCGGAAACCTAGCCATAGCT
1981 ---------+---------+---------+---------+---------+---------+---------+ 2040
TACGACAGGTTACTGGGGTATGGAGGATAACACCCTTTGTCGCCTTTGGATCGGTATCGA
a M L S N D P I P P I V G N S G N L A I A -

FIG.4I

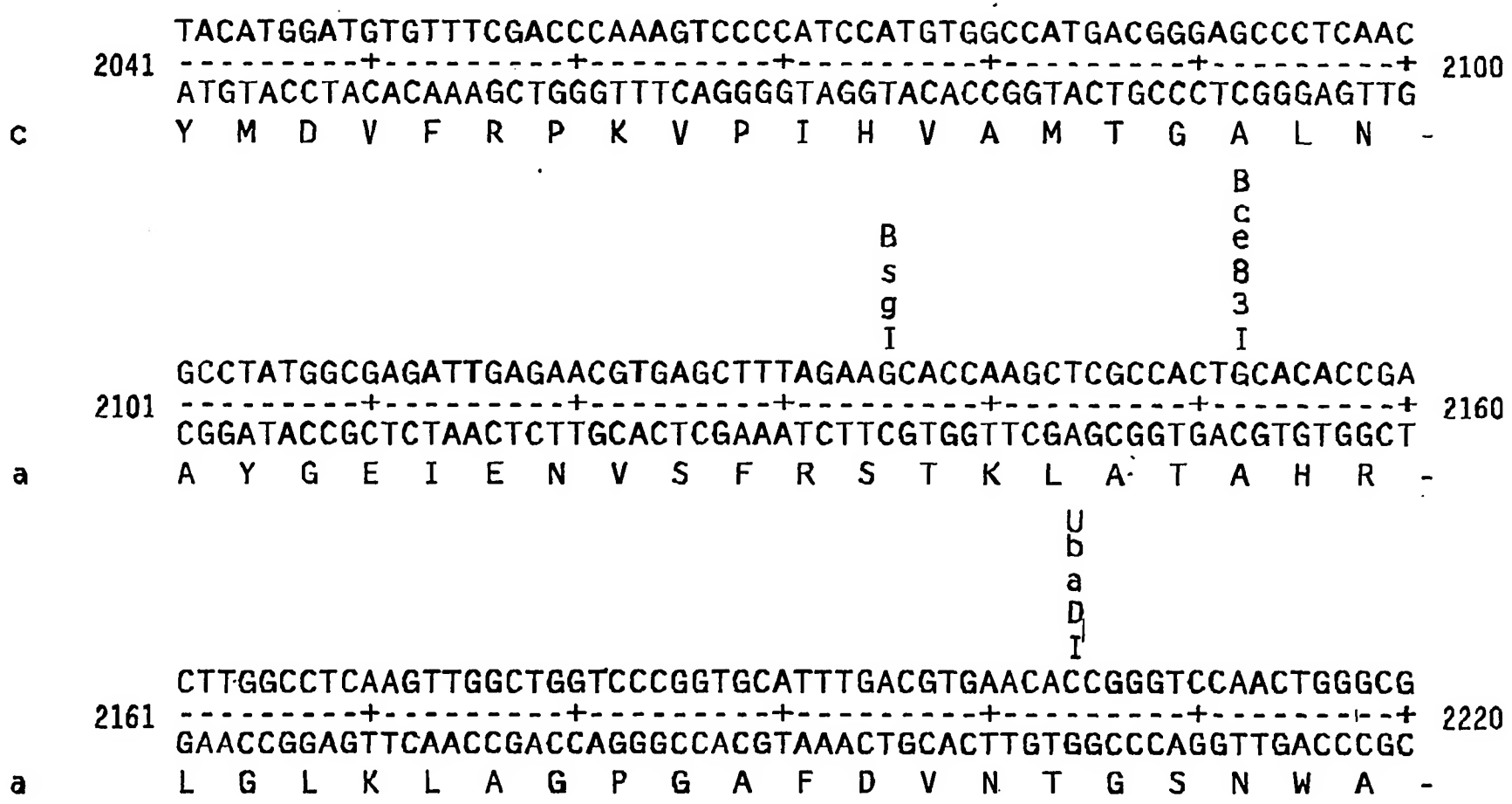

TACATGGATGTGTTTCGACCCAAAGTCCCCATCCATGTGGCCATGACGGGAGCCCTCAAC
2041 2100
ATGTACCTACACAAAGCTGGGTTTCAGGGGTAGGTACACCGGTACTGCCCTCGGGAGTTG
c Y M D V F R P K V P I H V A M T G A L N -
BsgI
BceA83I
GCCTATGGCGAGATTGAGAACGTGAGCTTTAGAAGCACCAAGCTCGCCACTGCACACCGA
2101 2160
CGGATACCGCTCTAACTCTTGCACTCGAAATCTTCGTGGTTCGAGCGGTGACGTGTGGCT
a A Y G E I E N V S F R S T K L A T A H R -
UbaDI
CTTGGCCTCAAGTTGGCTGGTCCCGGTGCATTTGACGTGAACACCGGGTCCAACTGGGCG
2161 2220
GAACCGGAGTTCAACCGACCAGGGCCACGTAAACTGCACTTGTGGCCCAGGTTGACCCGC
a L G L K L A G P G A F D V N T G S N W A -

FIG.4J

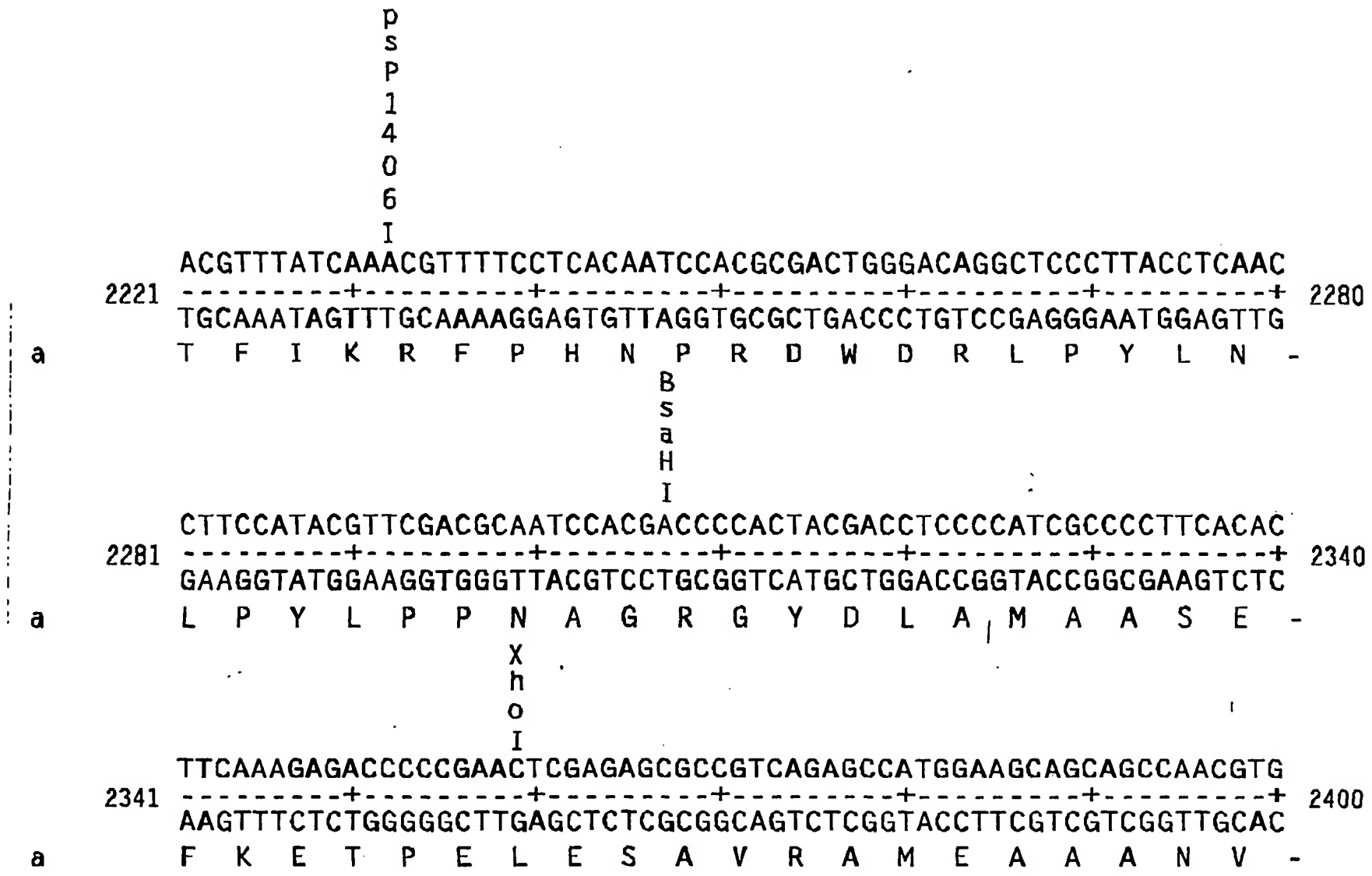

PspI406I
ACGTTTATCAAACGTTTTCCTCACAATCCACGCGACTGGGACAGGCTCCCTTACCTCAAC
2221 2280
TGCAAATAGTTTGCAAAAGGAGTGTTAGGTGCGCTGACCCTGTCCGAGGGAATGGAGTTG
a T F I K R F P H N P R D W D R L P Y L N -
BsaHI
CTTCCATACGTTCGACGCAATCCACGACCCCACTACGACCTCCCCATCGCCCCTTCACAC
2281 2340
GAAGGTATGGAAGGTGGGTTACGTCCTGCGGTCATGCTGGACCGGTACCGGCGAAGTCTC
a L P Y L P P N A G R G Y D L A M A A S E -
XhoI
TTCAAAGAGACCCCCGAACTCGAGAGCGCCGTCAGAGCCATGGAAGCAGCAGCCAACGTG
2341 2400
AAGTTTCTCTGGGGGCTTGAGCTCTCGCGGCAGTCTCGGTACCTTCGTCGTCGGTTGCAC
a F K E T P E L E S A V R A M E A A A N V -

FIG.4K

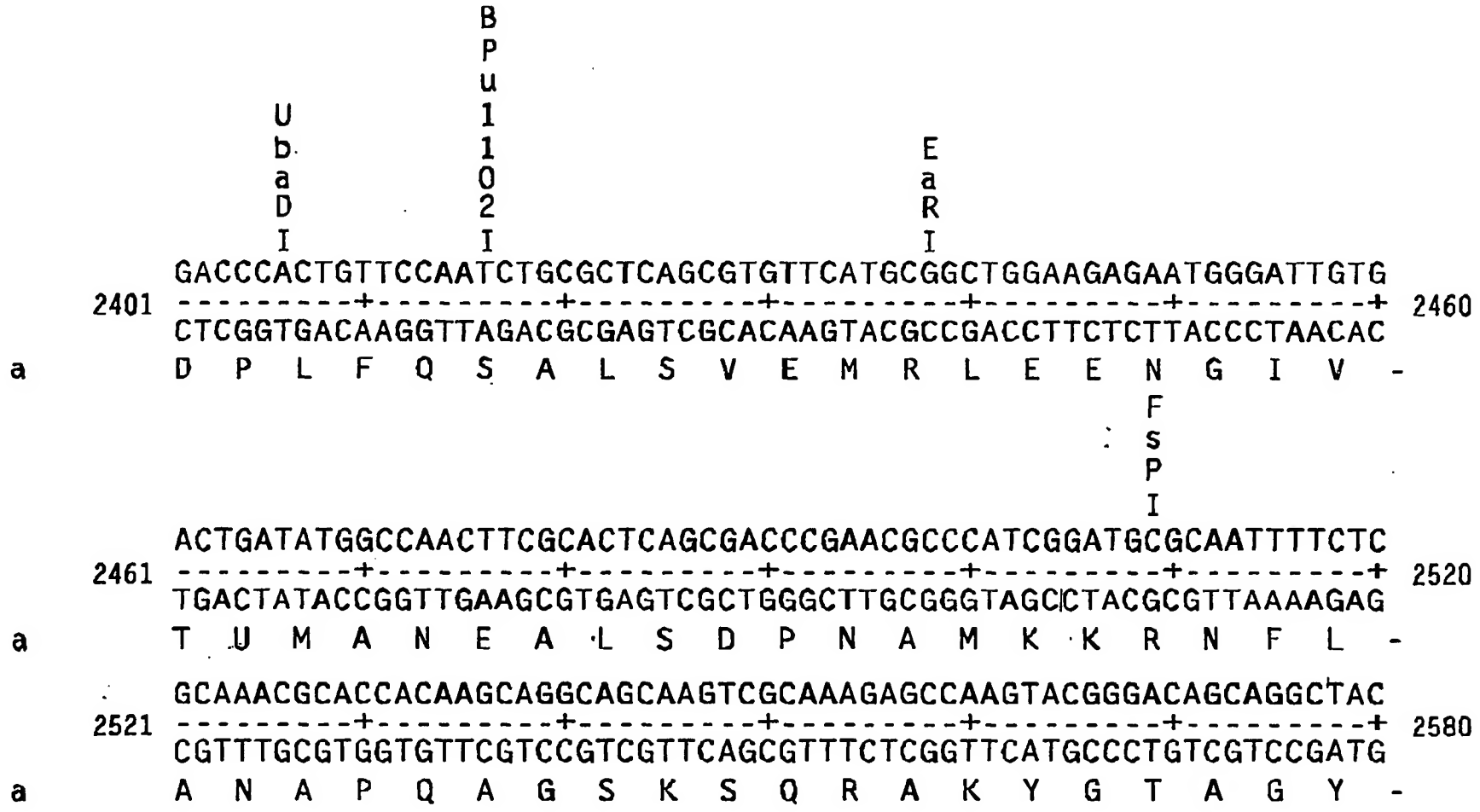

BpuI102I
UbaDI
EaRI
GACCCACTGTTCCAATCTGCGCTCAGCGTGTTCATGCGGCTGGAAGAGAATGGGATTGTG
2401 + 2460
CTCGGTGACAAGGTTAGACGCGAGTCGCACAAGTACGCCGACCTTCTCTTACCCTAACAC
a D P L F Q S A L S V E M R L E E N G I V -
FspI
ACTGATATGGCCAACTTCGCACTCAGCGACCCGAACGCCCATCGGATGCGCAATTTTCTC
2461 + 2520
TGACTATACCGGTTGAAGCGTGAGTCGCTGGGCTTGCGGGTAGCCTACGCGTTAAAAGAG
a T U M A N E A L S D P N A M K K R N F L -
GCAAACGCACCACAAGCAGGCAGCAAGTCGCAAAGAGCCAAGTACGGGACAGCAGGCTAC
2521 + 2580
CGTTTGCGTGGTGTTCGTCCGTCGTTCAGCGTTTCTCGGTTCATGCCCTGTCGTCCGATG
a A N A P Q A G S K S Q R A K Y G T A G Y -

FIG.4L

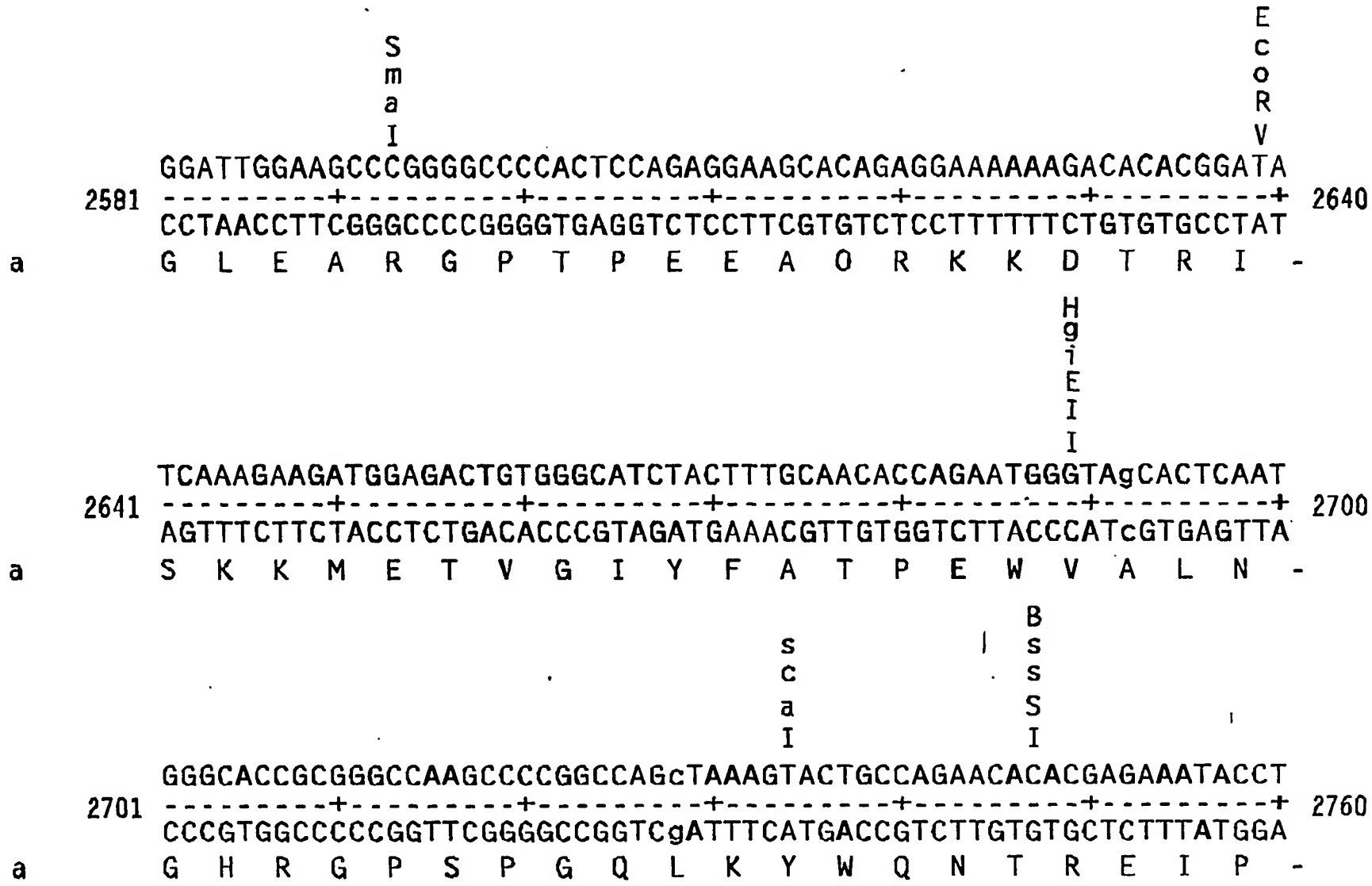

SmaI
EcoRV
GGATTGGAAGCCCGGGGCCCCACTCCAGAGGAAGCACAGAGGAAAAAAGACACACGGATA
2581 2640
CCTAACCTTCGGGCCCCGGGGTGAGGTCTCCTTCGTGTCTCCTTTTTTCTGTGTGCCTAT
a G L E A R G P T P E E A O R K K D T R I -
HgiEII
TCAAAGAAGATGGAGACTGTGGGCATCTACTTTGCAACACCAGAATGGGTAgCACTCAAT
2641 2700
AGTTTCTTCTACCTCTGACACCCGTAGATGAAACGTTGTGGTCTTACCCATcGTGAGTTA
a S K K M E T V G I Y F A T P E W V A L N -
ScaI
BssSI
GGGCACCGCGGGCCAAGCCCCGGCCAGcTAAAGTACTGCCAGAACACACGAGAAATACCT
2701 2760
CCCGTGGCCCCCGGTTCGGGGCCGGTCgATTTCATGACCGTCTTGTGTGCTCTTTATGGA
a G H R G P S P G Q L K Y W Q N T R E I P -

FIG.4M

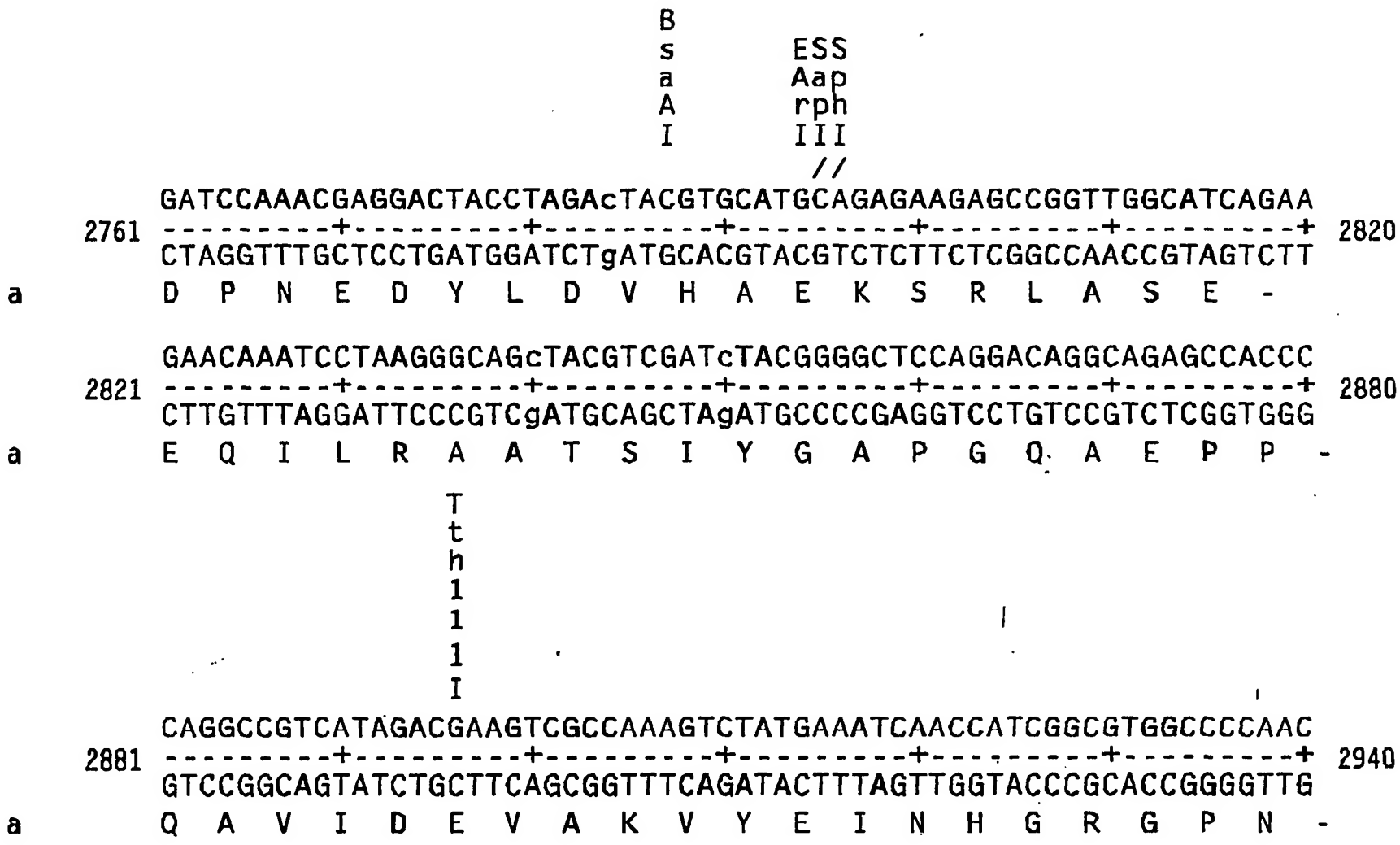

BsaAI
ESSAaphIII
//
GATCCAAACGAGGACTACCTAGAcTACGTGCATGCAGAGAAGAGCCGGTTGGCATCAGAA
2761 2820
CTAGGTTTGCTCCTGATGGATCTgATGCACGTACGTCTCTTCTCGGCCAACCGTAGTCTT
a D P N E D Y L D V H A E K S R L A S E -
GAACAAATCCTAAGGGCAGcTACGTCGATcTACGGGGCTCCAGGACAGGCAGAGCCACCC
2821 2880
CTTGTTTAGGATTCCCGTCgATGCAGCTAgATGCCCCGAGGTCCTGTCCGTCTCGGTGGG
a E Q I L R A A T S I Y G A P G Q A E P P -
TTth1111I
CAGGCCGTCATAGACGAAGTCGCCAAAGTCTATGAAATCAACCATCGGCGTGGCCCCAAC
2881 2940
GTCCGGCAGTATCTGCTTCAGCGGTTTCAGATACTTTAGTTGGTACCCGCACCGGGGTTG
a Q A V I D E V A K V Y E I N H G R G P N -

FIG.4N

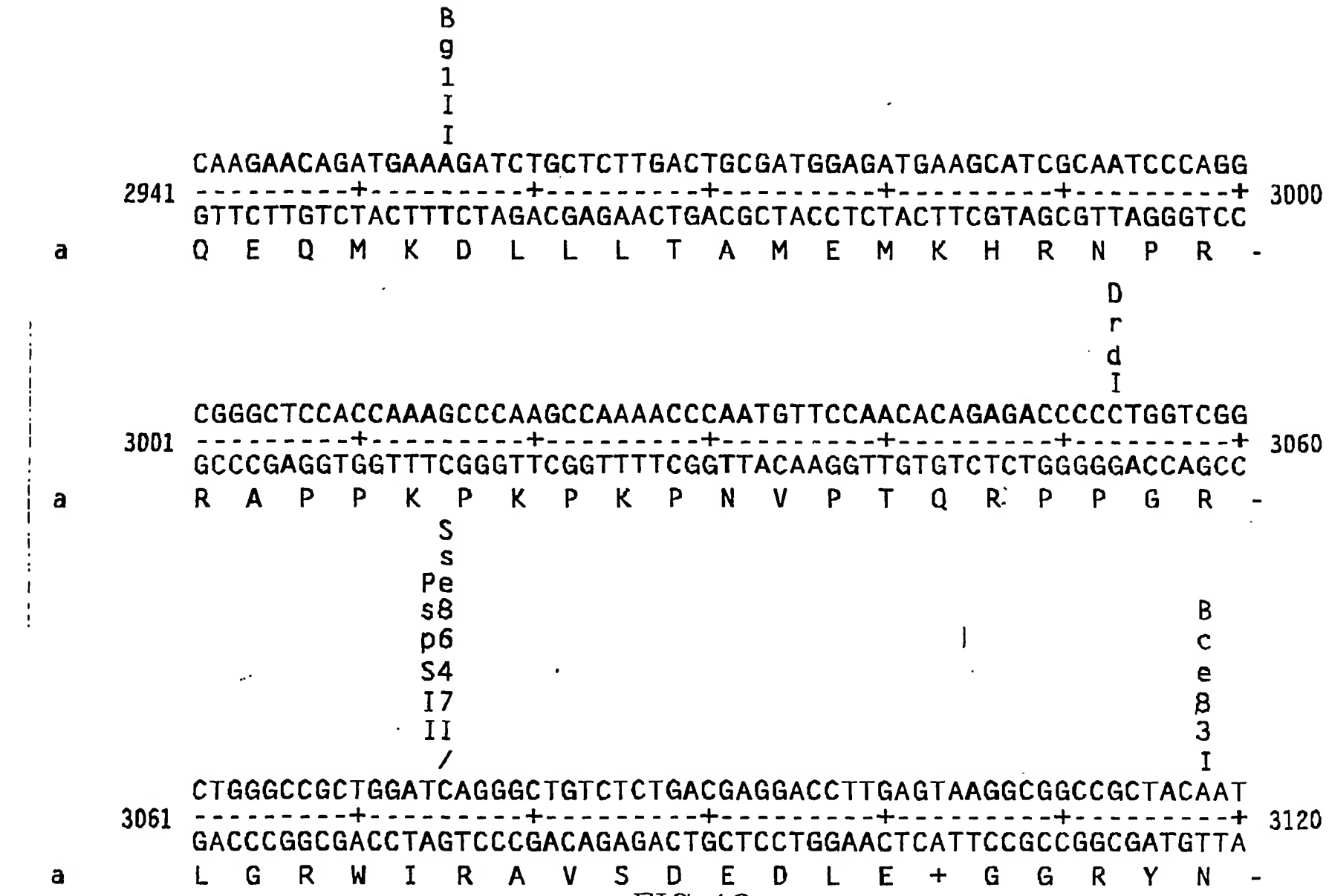

B
g
1
I
I
CAAGAACAGATGAAAGATCTGCTCTTGACTGCGATGGAGATGAAGCATCGCAATCCCAGG
2941 ---------+---------+---------+---------+---------+---------+---------+ 3000
GTTCTTGTCTACTTTCTAGACGAGAACTGACGCTACCTCTACTTCGTAGCGTTAGGGTCC
a Q E Q M K D L L L T A M E M K H R N P R -
D
r
d
I
CGGGCTCCACCAAAGCCCAAGCCAAAACCCAATGTTCCAACACAGAGACCCCCTGGTCGG
3001 ---------+---------+---------+---------+---------+---------+---------+ 3060
GCCCGAGGTGGTTTCGGGTTCGGTTTTCGGTTACAAGGTTGTGTCTCTGGGGGACCAGCC
a R A P P K P K P K P N V P T Q R P P G R -
S
s
Pe
s8
p6
S4
I7
II
/
B
c
e
8
3
I
CTGGGCCGCTGGATCAGGGCTGTCTCTGACGAGGACCTTGAGTAAGGCGGCCGCTACAAT
3061 ---------+---------+---------+---------+---------+---------+---------+ 3120
GACCCGGCGACCTAGTCCCGACAGAGACTGCTCCTGGAACTCATTCCGCCGGCGATGTTA
a L G R W I R A V S D E D L E + G G R Y N -

FIG.40

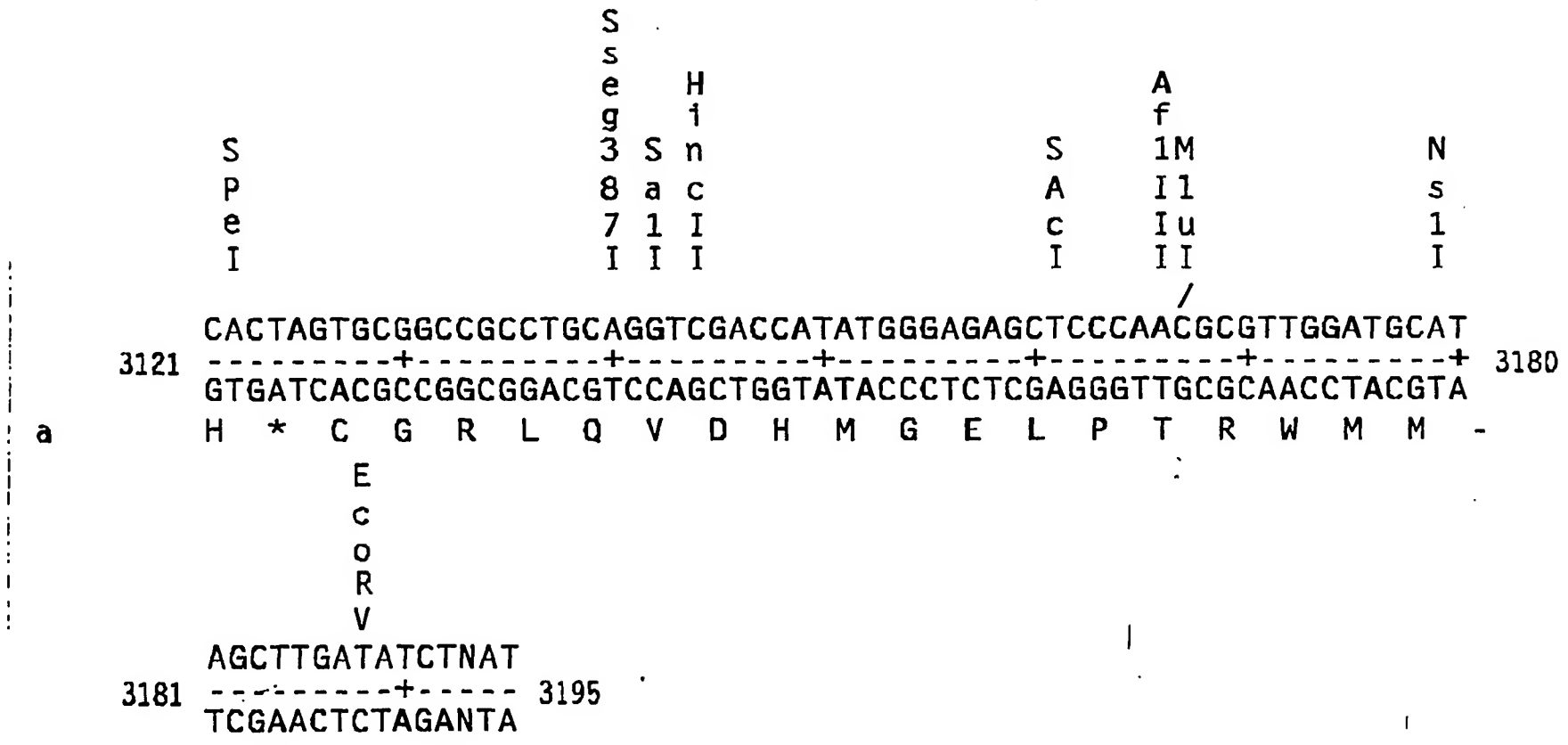

S
s
e
g
H
i
A
f
S
3
S
n
S
1M
N
P
8
a
c
A
I1
s
e
7
1
I
c
Iu
1
I
I
I
I
I
I
/
CACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCAT
3121 ---------+---------+---------+---------+---------+---------+---------+ 3180
GTGATCACGCCGGCGGACGTCCAGCTGGTATACCCTCTCGAGGGTTGCGCAACCTACGTA
a H * C G R L Q V D H M G E L P T R W M M -
E
c
o
R
V
AGCTTGATATCTNAT
3181 ---------+----- 3195
TCGAACTCTAGANTA

FIG.4P

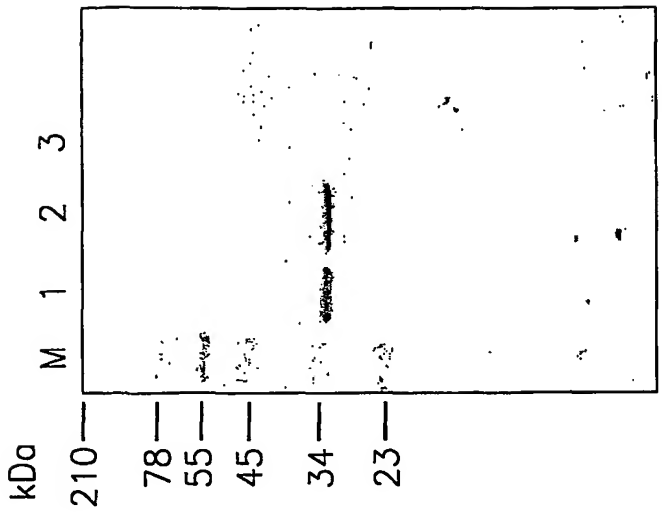


FIG.5C

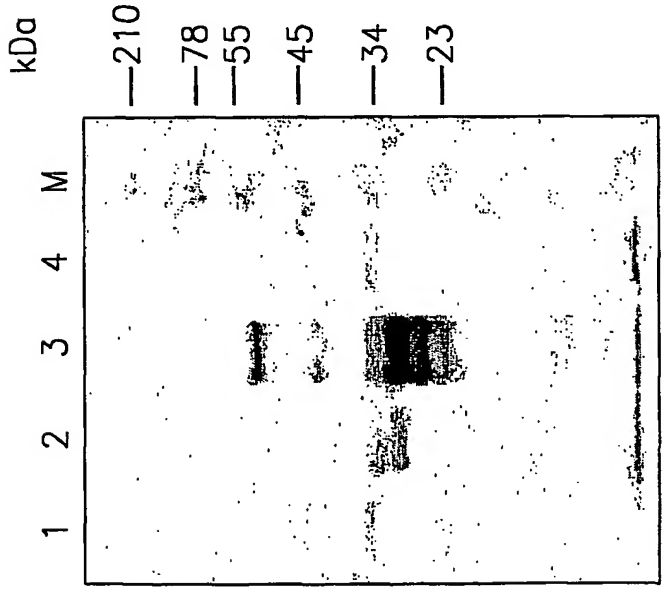


FIG.5B

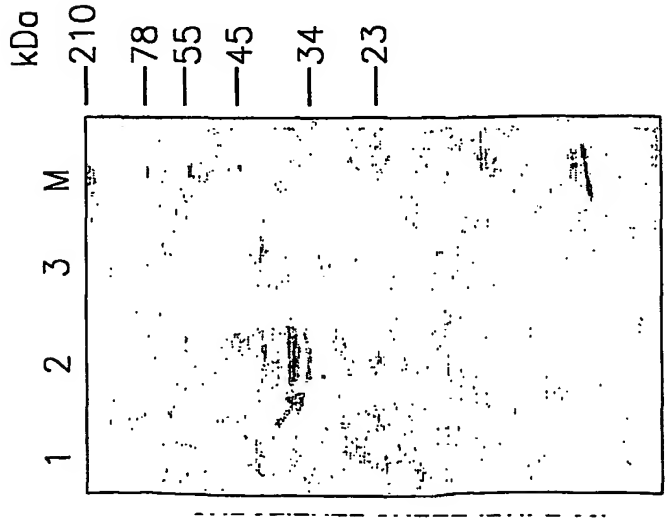
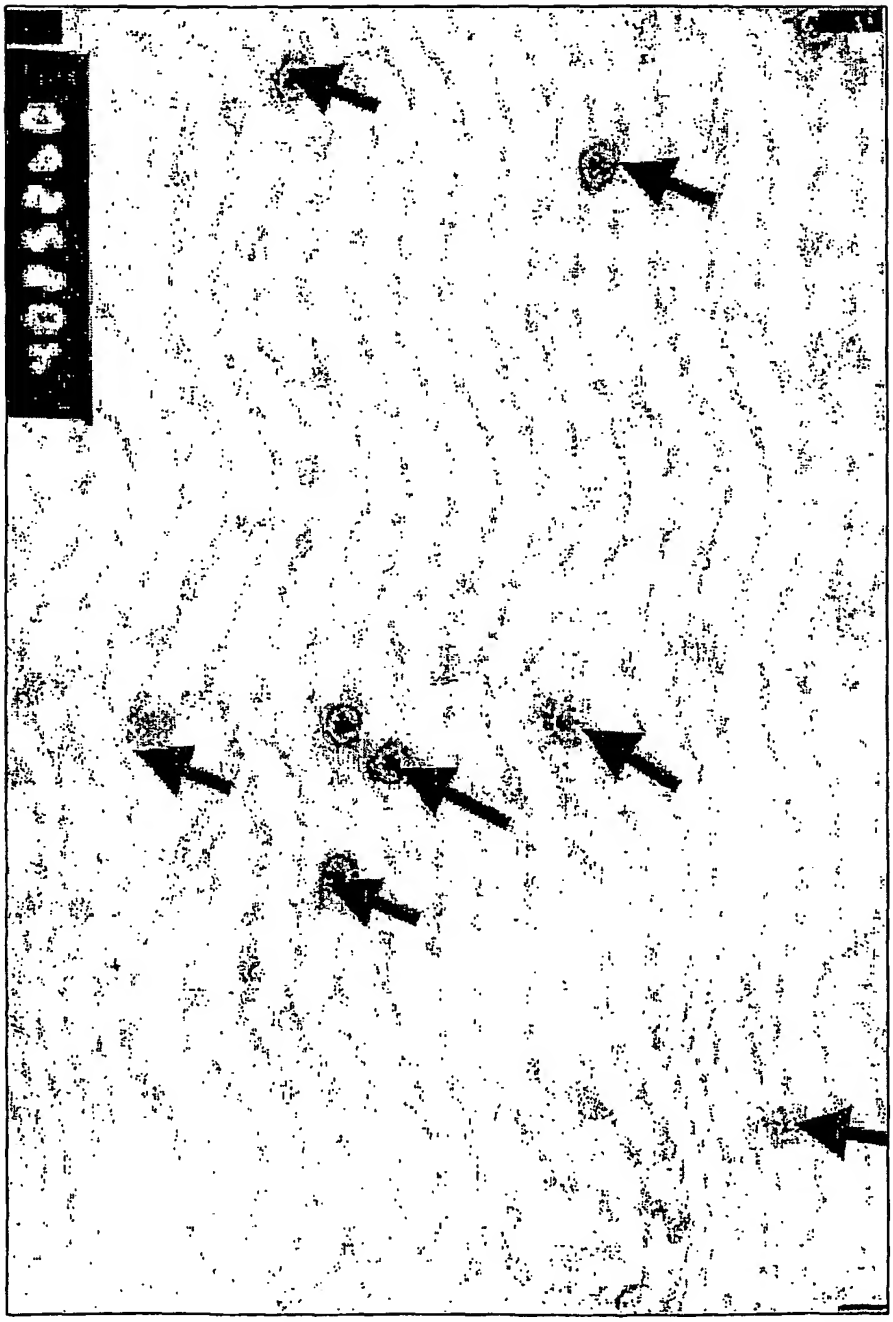


FIG.5A



RECOMBINANT IBDV PARTICLES IN E. COLI CELLS

FIG.6

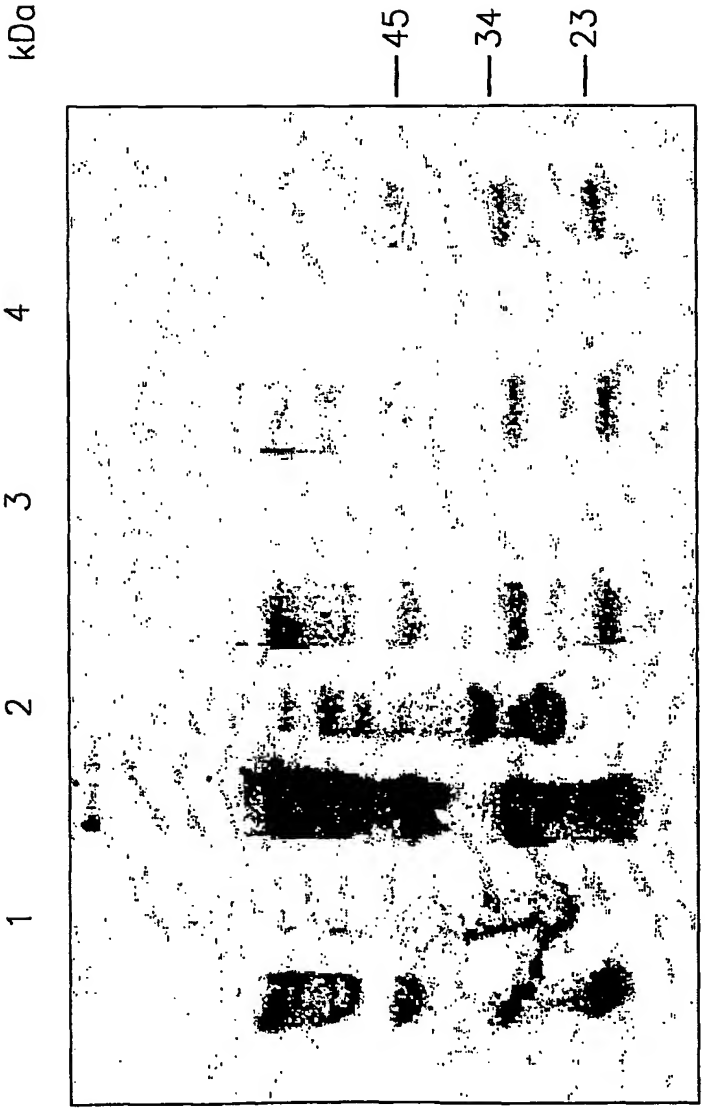
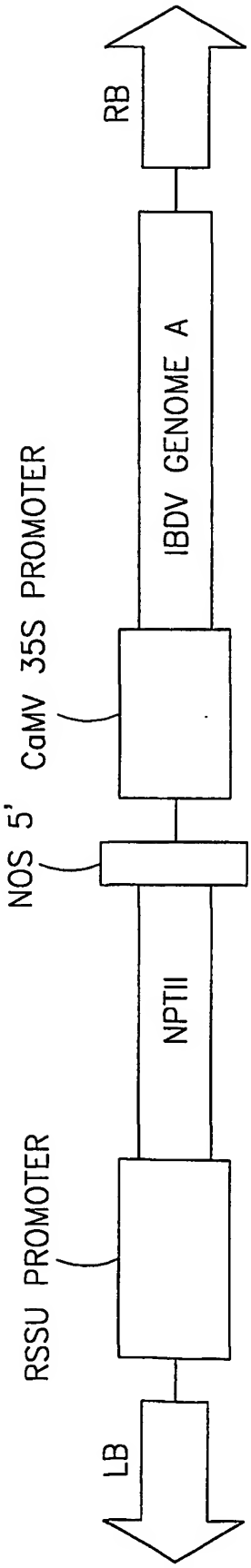


FIG.7

FIG.8



LB–LEFT BORDER
RB–RIGHT BORDER
RSSU–RUBISCO SMALL SUBUNIT
NPTII–NEOMYCIN PHOSPHO–TRANSFERASE GENE
CaMV35S–CAULIFLOWER MOZAIC VIRUS 35S PROMOTER

FIG.9

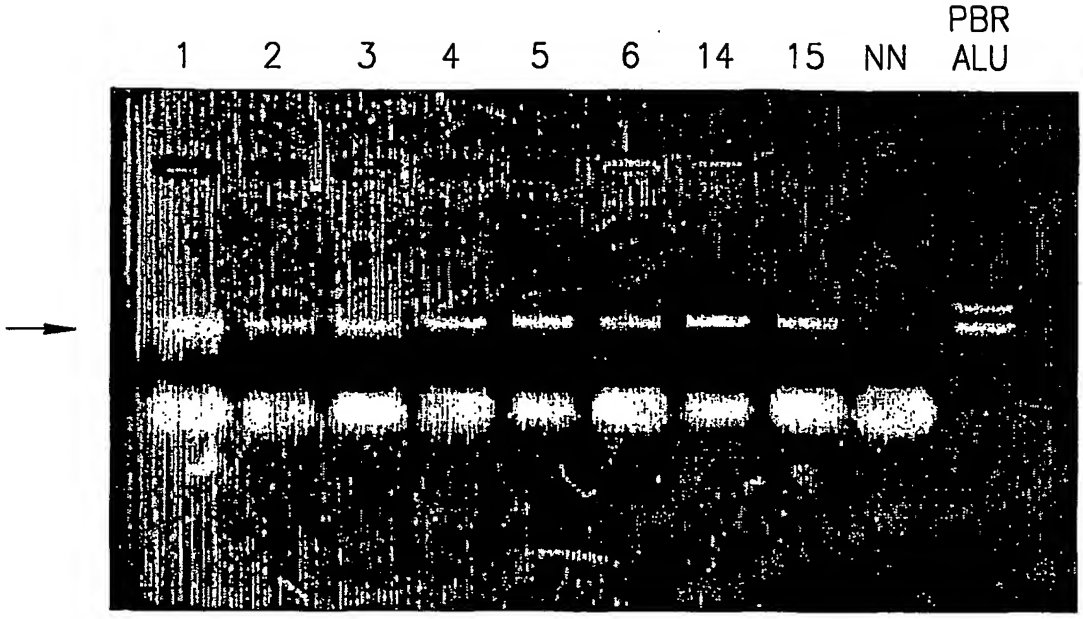


FIG.10A

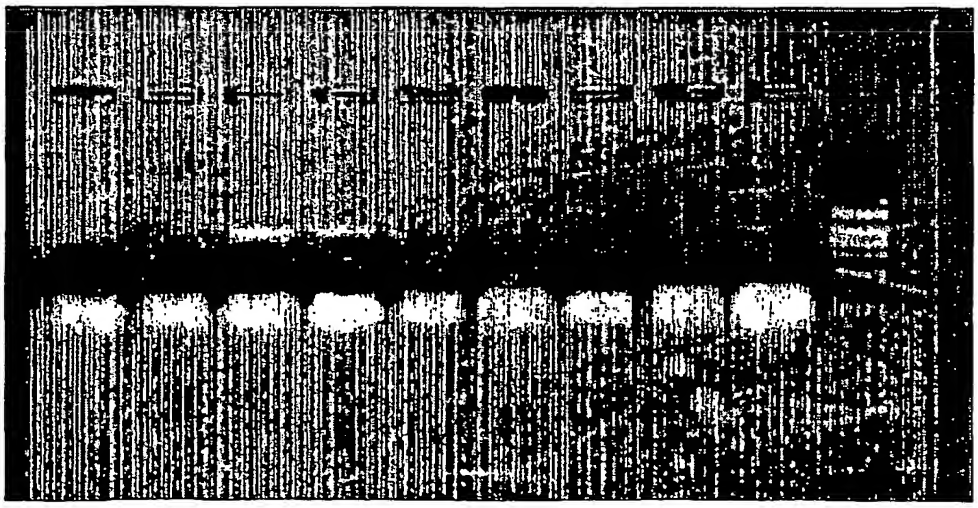


FIG.10B

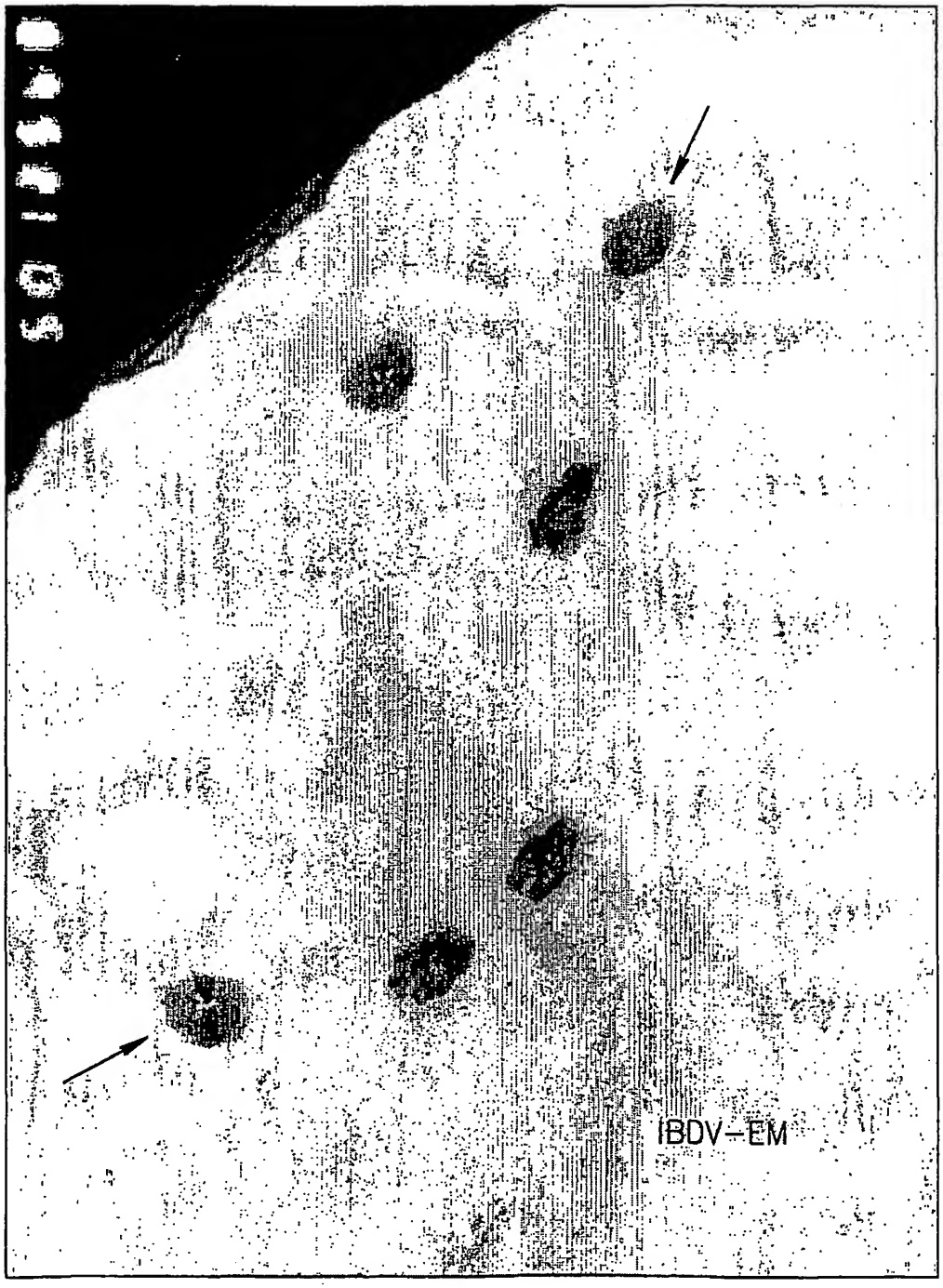


FIG.11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00360

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 39/12; C07H 21/02; C07K 14/08; C12N 7/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/186.1, 200.1, 204.1, 257.1, 93.2, 93.7; 435/69.3, 236, 414, 417, 419, 252.33, 800/288, 317.2, 317.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: transgenic, plant, bursa?, vaccine, birnavir?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,956,282 A (GOODMAN et al) 11 September 1990, see entire document.	1-10, 17, 19, 20
Y	US 5,605,827 A (JACKWOOD et al) 25 February 1997, see entire document.	1-20
Y	US 5,641,490 A (PAOLETTI et al) 24 June 1997, see entire document.	1-20
Y	US 5,788,970 A (VAKHARIA et al) 04 August 1998, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
29 NOVEMBER 2000	03 APR 2001

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PHUONG BUI
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

TERRY J. DEY
PARALEGAL SPECIALIST
TECHNOLOGY CENTER 1600

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00360

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00360

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/186.1, 200.1, 204.1, 257.1, 93.2, 93.7; 435/69.3, 236, 414, 417, 419, 252.33, 800/288, 317.2, 317.3

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-10, 17, 19 and 20, drawn to a transgenic plant and method of immunizing using said transgenic plant.
Group II, claim(s) 11-16 and 18-20, drawn to a bacteria vector and method of immunizing using said bacteria vector.

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the patentability of the transgenic plant does not depend on the patentability of the bacteria vector. Further, immunogenic IBDV proteins were known in the prior art (USPN 5605827, USPN 5788970, USPN 5641490).